PhD in Science and Technologies for the Earth and Environment (STAT)

Curriculum: Biology applied to Agriculture and Environment

Phytochemical Composition And Biological Activity Of Extracts From Lebanese Medicinal Plants

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➢ INTRODUCTION

Mediterranean Diet (MD) is considered one of the healthiest dietary habit [Serra-Majem et al., 2019]. It is possibly the best-studied and most evidence-based diet to prevent many diseases including non-alcoholic fatty liver diseases (NAFLD), obesity, diabetes, inflammation and cancer [Al Shaikh et al., 2019]. MD is characterized by high intake of vegetables and fruits resulted to provide a high amount of fibers, vitamins and antioxidants, such as phenolic compounds (PC) which may be responsible of its protective and beneficial effects. Many evidences suggest that higher adherence to the MD is associated with lower risk of mortality, cardiovascular disease, metabolic disease, and cancer [Schwingshackl et al. 2019].

The Lebanon covers a total of 10,452 km^2 in the eastern Mediterranean and it presents a climatic and ecological diversity that is unique among other countries of the region. For this reason, Lebanon is recognized as a center of plant biodiversity [Baydoun et al., 2015]. The 4th Lebanon national report on Conventional Biological Diversity [MoE et al. 2009, 2011] described that Lebanon hosts over 2,600 terrestrial plant species with a high rate of endemism (12%) including 8.5% as broad endemics (Lebanon, Syria, and Palestine) and 3.5% as narrow endemics to Lebanon. Wild edible plants have remarkable roles in Lebanese diet and the utilization of wild vegetables as a nutritional source is diffuse among local people. Indeed, despite the increasing use of synthetic chemical drugs, in Lebanon, natural products and local medicinal plants including herbal tea, herbal formulation and herbalist preparation continued to be the first 'treatment of choice' for different diseases such as headaches, stomachaches, abdominal pain, microbial infections, asthma, coughs, pulmonary and urinary disorders [Loizzo et al., 2008]. *"If it does not benefit, it would not harm"* is the common notion among many Lebanese people who heavily use plant in traditional medicine [Gali-Muhtasib et al., 2000].

The phenolic compounds are the most important bioactive compounds of the medical plants and have been investigated as candidates to prevent various diseases [Kaulmann and Bohn, 2016; García-Conesa, 2017]. PC are characterized by at least one aromatic ring attached to hydroxyl groups. PC are classified in two general classes: flavonoids and phenolic acids. Flavonoids are

further divided into flavones, flavononse, flavonols, flavanols, isoflavones. Phenolic acids are classified into hydroxybenzoic and hydroxycinnamic acids.

Plants have been an important source for pharmacologically active compounds. Nowadays, the interest in bioactive compounds from plants is still great also for the increasing prevalence of chronic diseases worldwide. Most chronic diseases are usually complex disorders where multiple are the causes and the mechanisms. Non-alcoholic fatty liver disease (NAFLD) is the most important chronic hepatic disease which heavily contributes to the obesity-related morbidity and mortality. Several factors are involved in the pathologic mechanisms that ultimately lead to the accumulation of hepatic fat and NAFLD progression (from simple steatosis to NASH, till to cirrhosis and hepatocellular carcinoma). Moreover, consolidated data and evidences indicate that oxidative stress and inflammation may play a critical role in the development of obesity-triggered NAFLD, Hence, antioxidants are frequently used to treat oxidative liver injury, and the consumption of antioxidants is known to be an important means of preventing or delaying the appearance of liver diseases.

Cancer is a generic term for a large group of diseases that can affect any part of the body. Cancer represents a group of diseases characterized by the uncontrolled growth, proliferation and spread of ‘abnormal’ cells in the body. Even with combination treatment regimens, survival rates of many type of cancer remain low and cancer is still a leading cause of death worldwide, accounting for an estimated 9.6 million deaths in 2018. The uncontrolled proliferation of cancer cells mainly originates from their ability to avoid programmed cell death, so-called apoptosis [Jan and Chaudhry, 2019], that is why induction of apoptosis in cancer has been identified as a target for treatment of cancer. Novel bioactive compounds from plants could improve the cancer therapy by both reducing the side effects of common chemotherapeutics and by act in synergy with them.

In summary, the Mediterranean area especially the eastern part including Lebanon, is rich in term of plant biodiversity, including high valuable medicinal plants that are largely used traditionally in herbal medicine, these plants represent an important source of plant-derived bioactive compounds, besides, there is a need to characterize these compounds, evaluate their bioactivity and clarify scientifically the use of these plant as traditional and alternative medicine, lack of these information may limit their use and studying them may improve the traditional use, discovery of new natural bioactive compounds and finally formulate new plant-based

nutraceutical and therapeutically agents. In this context, the aim of my PhD thesis, and as a part of our ongoing program in the medicinal plants filed, was the characterization of a pool of phytochemical compounds with a special focus on polyphenols isolated from different medicinal plants from the Lebanese flora, using analytical tools (as described later in the chapters) including high-performance liquid chromatography (HPLC) coupled with different detectors such as UV-visible and electrospray ioniziation (ESI) Mass spectrometry (MS), following by the study of their biological activities using different *in vitro* cellular well-established model for several chronic diseases. The plants that I choose for my study have a significant value in the traditional medicine in Lebanon, where they are employed as a food but also in folk medicine to treat/prevent many illnesses. I employed different cellular models of oxidative stress and related chronic diseases I cited above (NAFLD, inflammation and cancer).

These plants as mentioned above are employed in many food and herbal tea preparations, local people in Lebanon use it largely, often daily, in their food, especially *Thymbra spicata* and *Rhus coriaria* as herbal mixture called Za'atar. Before choosing this plant for the PhD project, we have intensely studied the significant of 30 Lebanese plants in two ways: 1) Investigating the traditionally use of the chosen plants; 2) Studying the previous work on the chemical and biological activity of these plants. We conclude that some of these plants could have a significant bioactivity regarding their important use in traditional medicine, and no study was done befor for testing their activity in our cellular model of different chronic diseases, then, we have proceeded the project aimed to explore the chemical and biological activities of three selected plants.

The plants I included in my study were:

- ***Thymbra spicata:*** an aromatic plant of the *Lamiaceae* family which includes many thyme-like plants. In Lebanon it is known with the popular name of "*Zaa'tar*" (a mixture largely employed as food in salads and spices), and it finds applications in traditional medicine to treat/prevent many diseases such as cramps, muscle pains, hyperglycemia and inflammation. Aromatic plants, *Lamiacieae* especially, have been used in the Middle East for long time for their preservative and medicinal properties; their extracts and essential oils are rich in phenolic volatile compound such as monoterpenes phenols

[Mamadalieva et al., 2017]. In my thesis work I prepared two extracts (aqueous and ethanolic) using aerial parts of *T. spicata* collected from *Marrakeh* in South Lebanon. HPLC/MS was used to explore the phenolic composition of both extracts showing that they were rich in PC, with a different PC profile depending on the solvent. These two extracts were investigated in two research lines:

- *(i)* at low doses (1.5 µg/mL) the extracts were tested for their possible anti-steatotic and anti-oxidant activities in well-established *in vitro* cellular models of hepatic steatosis and atherosclerosis.
- (ii) at higher doses (50 & 100 µg/mL), the extracts were tested for their possible cytotoxic and antiproliferative activities using different cancer cells lines being representative of important human tumors.

- ***Ephedra foeminea*:** a low stalky Eurasian shrub belonging to the *Ephedraceae* family. It is mentioned as *Alanda* in traditional Arab, and it is ubiquitous in the southeastern Mediterranean. Unlike other *Ephedra* species, little is known about *E. foeminea* phytochemical constituents and poor is its use as popular medical remedy. *E. foeminea* it has only been mentioned in folk medicine for treatment of anxiety and skin rash [Ben-Arye et al. 2016], and only recently some evidences have been reported for its potential use in cancer treatment [Ali-Shtayeh et al. 2016]. During my thesis work I prepared different extracts of *E. foeminea* fruits collected from *Marrake* in South Lebanon using different extraction solvents and methods. The extracts were subjected to HPLC/MS to identify their phytochemical constituents which are different depending on the polarity of the solvents. The extracts have been tested for their *in vitro* radical scavenging activity and *in vitro* antioxidant and cytoprotective activity using endothelial cells.

- ***Rhus coriaera L.* (Sumac):** a wild edible plant of *Anacardiaceae* family growing in the Mediterranean region. The dried fruit are the most commonly consumed part of this plant which is typically used as a condiment, spice, sauce, and appetizer. In folk medicine this plant has been used in the treatment of stroke, diarrhoea, hypertension, haematemesis, ophthalmia, stomach ache, diabetes, atherosclerosis, and liver disease. Furthermore, many

in vitro and *in vivo* studies suggest that *R. coriaria* might possess protective, antiseptic, antifungal, antibacterial, antioxidant, hypouricemic, hypoglycaemic and hepatoprotective properties [Anwer et al., 2013]. I tested two extracts from *R. coriaera* fruits collected from South Lebanon (aqueous and ethanolic) for their possible anti-inflammatory and anti-oxidant effects on microglial cells.

All the plants studied in this work were identified by Dr. George Tohme, Professor of Taxonomy and President of the National Council for Scientific Research (CNRS), Beirut, Lebanon in collaboration with Prof Mario Mariotti (DSTAV, University of Genoa- Italy). A voucher specimen for each plant has been deposited in the Herbarium of the Faculty of Science, Lebanese University, Hadath, Beirut, Lebanon.

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➢ CHAPTER 1.

Antisteatotic and antioxidant activities of *Thymbra spicata L.* Extracts in hepatic and endothelial cells as *in vitro* models of non-alcoholic fatty liver disease

This work has been published as research paper in *journal of Ethnopharmacology*

ABSTRACT

Ethnopharmacological relevance: *Thymbra spicata*, a member of the Lamiaceae family, is native to eastern Mediterranean area. Leaves of this plant are rich in phenolic compounds and are a popular remedy of traditional medicine in Lebanon to prevent and/or counteract hyperlipidemia and hyperglycemia.

Aim of the study: To evaluate the antisteatotic and antioxidant activities of extracts from leaves of *Thymbra spicata L*. using *in vitro* models of non-alcoholic fatty liver disease (NAFLD), a leading cause of liver-related morbidity and mortality worldwide, for whom no effective treatments are still available.

Materials and Methods. Two different extracts from *Thymbra spicata L*. aerial parts were prepared using water (TW) or ethanol (TE) as solvent. Their chemical composition was characterized by gas and liquid chromatography coupled with mass spectrometry. Both extracts were tested on cultured hepatic and endothelial cells treated to mimic *in vitro* a multisistemic pathology such as NAFLD. We assayed the effects on lipid accumulation, free radical production, lipid peroxidation, cell migration.

Results. Both the total phenolic and the total flavonoid contents were higher in the ethanolic extract. Rosmarinic acid was the most abundant polyphenol in TW, while TE was richer in carvacrol. Our findings demonstrated that both extracts ameliorated lipid accumulation, oxidative stress and inflammation in the NAFLD cellular models. However, the aqueous extract was more effective to reduce hepatic steatosis, and the ethanolic extract had higheranti-oxidant potential and wound healing activity.

Conclusions.. *T. spicata* extracts could be promising bioactive products to develop natural therapeutic agents or dietary supplements to treat NAFLD and obesity-related metabolic disease. Our findings suggest that while the ethanolic extract might be used in preventing endothelium dysfunction, the aqueous extract would act better as lipid-lowering agent.

INTRODUCTION

The number of studies regarding the functional molecules in food which can give health benefits and promote well-being has greatly increased [Abuajah et al. 2015]. Phenolic compounds (PC) are the most numerous and widespread group of functional molecules that are found largely in the fruits, vegetables, cereals and beverages [Lobo et al. 2010]. Pleiotropic health beneficial effects have been described for dietary plant phenolic compounds [Pandey and Rizvi 2009]. Although the antioxidant activity is the most relevant biological function of PC which protect biological systems against the excess of "reactive oxygen species" (ROS), other biological effects have been reported, especially on the liver [Itoh et al. 2010, Rafiei et al. 2019]. Epidemiological studies have repeatedly shown an inverse association between the risk of chronic human diseases and the consumption of polyphenolic rich diet [Scalbert et al. 2005].

Plants are a source of bioactive compounds that might find application in developing of novel drugs. In Mediterranean area, thyme-like plants such as *Thymbra spicata* are widely diffused; their leaves and flowers are used in salad and tea infusion, and its components are employed in food industry for flavouring or as antimicrobial and antifungal agents [Bozkurt 2006, Eruygur 2017]. *T. spicata*, known as "Za'tar" in Lebanon belongs to the Lamiaceae family and it is rich in PC including phenolic acids (rosmarinic acid), phenolic monoterpenoids (carvacrol, thymol) and flavonoids (both glycosides and aglycones) [Dorman et al. 2004, Hanci et al. 2003]. The use of *T. spicata* in traditional medicine in Lebanon is well documented: leaves of this plant have known an ever-wider use to combat hypercholesterolaemia [Baser et al. 1986], and anti-oxidant and anti-steatotic activities have been reported in HFD-fed mice [Akkol et al. 2009, Avci et al. 2006].

The phenolic compounds received growing attention among nutritionists as possible therapy in the prevention of metabolic and CVD diseases [Rengasamy et al. 2018]. Excess energy intake is associated with body weight gain, which can lead to obesity and other metabolic disorders including the non-alcoholic fatty liver disease (NAFLD), the most important chronic liver disease worldwide [Wouters et al. 2010]. The hallmark of NAFLD is hepatic steatosis consisting of excess accumulation of triglycerides (TGs) in the liver. Severe steatosis is associated to increased production of ROS leading to oxidative stress which promote a disease progression [Manns et al. 2010]. Indeed, NAFLD may range from simple steatosis to steatohepatitis (NASH) with inflammation and fibrosis, cirrhosis and hepatocellular carcinoma [Vergani et al. 2017]. NAFLD is a multisistemic disease where both hepatocytes and endothelial cells are involved, with metabolic disorders causing endothelial dysfunction which in turn worsen hepatic metabolism alterations. The main goal for NAFLD therapy is to reverse or prevent the progression of simple steatosis to liver fibrosis. To date, no effective pharmacological treatment is available for NAFLD, due to potential adverse effects of conventional pharmacological therapies. Therefore, a growing interest in identifying agents for treatment and/or prevention of NAFLD progression has been addressed on studying complementary therapies that employ both natural and safe products such as herbal medicine [Bagherniya et al. 2018]. PC are possible therapeutic interventions, because their hepatoprotective potential against liver damages promoted by the oxidative reactions triggering lipid peroxidation [Ferramosca et al. 2017, Rajput et al. 2018].

Our study focused on investigating the beneficial effects of extracts from leaves of *Thymbra spicata*. *L* being rich of bioactive phenolic compounds. We used rat hepatoma FaO cells exposed to a mixture of oleate/palmitate that represent an accepted *in vitro* model for hepatic steatosis

which was largely employed in previous studies [Vecchione et al. 2016, 2017]. We used also human endothelial HECV cells exposed to hydrogen peroxide, as an *in vitro* model of endothelium damage, which is characteristic of metabolic syndrome [Vergani et al. 2017]. On these *in vitro* models we assessed the anti-steatotic and anti-oxidant activities of both aqueous and ethanolic extracts from *T. spicata* aerial parts.

MATERIALS AND METHODS

2.1 Chemicals

All chemicals, unless otherwise indicated, were supplied by Sigma-Aldrich Corp. (Milan, Italy).

2.2 Collection of plant material and extraction

Aerial parts of *Thymbra spicata* were collected from flowering plants growing wild in *"Maarakeh"* south Lebanon, 280 m above sea level (33° 16′ 35.59″ N and 35° 19′ 02.89″). The plant was authenticated by Dr. George Tohme, Professor of Taxonomy and President of the National Council for Scientific Research (CNRS), Beirut, Lebanon. A voucher specimen (L1.125/1) has been deposited in the Herbarium of the Faculty of Science, Lebanese University, Hadath, Beirut, Lebanon. Following dissection, the aerial parts of *Thymbra spicata L*. were shade dried for three weeks at room temperature. Then, the grinded materials were extracted with two different solvents, ethanol (100%) or distilled water using standard procedures [Lupidi et al. 2011]. For aqueous extraction, 15 grams of dried material were dissolved in 250 ml of distilled water, and placed on stirrer for 3 hours at room temperature; and for the last 10 min at 60°C. For ethanolic extraction, 25 grams of dried material were dissolved in 300 ml of pure ethanol and placed on stirrer overnight at room temperature. At the end, both solvents were evaporated in a Rotavac vario power unit (Heidolph Instruments, Schwabach, Germany), and then freeze-dried

in Alpha 1-4 LD plus lyophilizer (CHRIST, Osterode am Harz, Germany). Then, the dry fractions were stored at 4°C until use.

2.3 Total Phenol Quantification

The total phenol content (TPC) was estimated according to the *Folin-Ciocalteu* method [Singleton, Rossi, 1965]. Briefly, 100 µl aliquots of each extract (1 mg/ml) were mixed with 0.5 ml of *Folin–Ciocalteau* reagent. After 5 min, 1.5 ml of Na_2CO_3 2% (w/v) were added, and the sample was incubated in the dark at room temperature for 30 min. The controls contained all the reaction reagents except the extract. The absorbance was measured at 760 nm using an U-2900 UV-Vis spectrophotometer 200 V (Hitachi High-Technologies, Japan) and compared to a gallic acid calibration curve. The results were expressed in mg of Gallic Acid Equivalents (GAE) per g of dry weight of plant powders.

2.4 Total Flavonoid Quantification

Total flavonoid content (TFC) was estimated by aluminium chloride colorimetric method according to Quettier-deleu et al [Quettier-Deleu et al. 2000]. Briefly, aliquots of 1 ml of extracts (0.5 mg/ml) were mixed with 1 ml of 2% (w/v) methanolic $AlCl_3$ solution. After incubation at room temperature in darkness for 15 min, the absorbance of all samples was determined at 415 nm using UV-Vis spectrophotometer and compared to a quercetin calibration curve. The blank contained all the reaction reagents except the extract. The results were expressed in mg of Quercetin Equivalent (QE) per g of dry weight of plant powders.

2.5 Chromatographic Analyses

2.5.1 GC–MS analysis Analytical gas chromatography (GC) was carried out using a GC FOCUS SERIES (Thermo Fisher Scientific, Milan-Italy) fitted with a GsBP-5MS capillary column (30 m×0.25 mm), 0.25 um film thickness. Helium was the carrier gas (0.7 ml/min). Column temperature was initially kept at 40°C for 5 min, then gradually increased to 85°C at 5°C/min rate, held for 20 min and finally raised to 300°C at 10°C/min. Aliquots (1 µl) of the diluted samples (1/100 v/v, in methanol) were injected at 250°C, in the splitless mode. The mass spectroscopy (MS) analyses were performed with a ISQ-MASS SPECTROMETER (Thermo Fisher Scientific), the source temperature was 310°C, the transfer-line temperature 320°C, the ionization voltage was 70 eV, and the mass range was 50–400 amu in the full scan mode. The identification and quantification of the different constituents in the extracts were based on the comparison of: (i) their retention indices (RIs), determined relatively to the retention time of n-alkanes (C8–C24) on capillary columns, compared with those reported in the literature or with those measured for pure compounds; (ii) their mass spectra with those listed in the commercial mass spectral libraries NIST, Wiley 275. The relative percentages of the components were calculated based on the GC-FID peak areas without using correction factors.

2.5.2 HPLC-MS analysis. High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS/MS) was performed using an Agilent 1100 HPLC-MSD Ion Trap XCT system, equipped with an electrospray ion source (HPLC-ESI-MS) (Agilent Technologies, Santa Clara, CA, USA). Separation of extracts was performed on a Jupiter C18 column 1 mm × 150 mm with 3.5 µm particle size (Phenomenex, USA). As eluents we used water (eluent A) and MeOH (eluent B), both added with 0.1% formic acid. The gradient employed was: 15% eluent B for 5 min, linear to 100% eluent B in 35 min, and finally hold at 100% eluent B for another 5 min. The flow rate was set to 50 µL/min with a column temperature of 30°C. The injection

volume was 8 μL. Ions were detected in the positive and negative ion mode, in the 100–800*m*/*z* range, and ion charged control with a target ion value of 100,000 and an accumulation time of 300 ms. A capillary voltage of 3300 V, nebulizer pressure of 20 psi, drying gas of 8 L/min, dry temperature of 325°C, and 2 rolling averages (averages: 5) were the parameters set for the MS detection. MS/MS analysis was conducted using an amplitude optimized time by time for each compound. From the chromatograms, the percentage of PC for each extract was calculated on the basis of the peak area.

2.6 Radical Scavenging Activity

The free radical scavenging capacity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [Brand-Williams et al. 1995] with slight modification. Briefly, 1 ml aliquots of extract at different concentrations were mixed with 1ml of DPPH solution (0.15 mM in ethanol) and incubated in darkness at room temperature for 30 min. Ascorbic acid was used as positive control. At the end, the absorbance at 517 nm was measured by UV-Vis spectrophotometer. Control sample contained all the reaction reagents except the extract. The percentage inhibition values were calculated using the following equation [Brand-Williams et al. 1995]:

$$\% \text{ Scavenging activity} = [(\text{Abs control} - \text{Abs sample})]/(\text{Abs control})] \times 100$$

and the IC50 values were estimated by a nonlinear regression algorithm.

2.7 Cell culture and treatments

FaO cells (European Collection of Authenticated Cell Cultures-ECACC- Salisbury, Wiltshire ,UK) are a rat hepatoma cell line maintaining hepatocyte-specific markers. Cells were grown in a humidified atmosphere with 5% CO_2 at 37°C in Coon's modified Ham's F12 medium supplemented with L-Glutamine and 10% foetal bovine serum (FBS) [Vergani et al. 2017].

HECV cells (Cell Bank and Culture-GMP-IST-Genoa, Italy) are a human endothelial cell line isolated from umbilical vein; they were grown at 37°C with 5% CO2 in Dulbecco's modified Eagle's medium High Glucose (D-MEM) supplemented with L-Glutamine and 10% FBS. For treatments, cells were grown until 80% confluence, then incubated overnight in serum-free medium with 0.25% bovine serum albumin (BSA). To mimic *in vitro* the effect of a high fat diet, FaO cells were treated for 3 h with a mixture of oleate/palmitate at a final concentration of 0.75 mM (2:1 molar ratio). To induce oxidative stress, HECV cells were incubated for 24 h with H2O2 (20nM). Thereafter, cells were incubated for 24h with either aqueous or ethanolic extracts at different concentrations (0.15, 1.5 and 15 μg/ml), for each experiment, treatment was performed in quadruplicates.

2.8 Protein quantification

The protein content was determined by the Bradford assay using BSA as a standard [Bradford, 1976].

2.9 Quantification of triglycerides

At indicate times, FaO cells were scraped, centrifuged and lysed and lipids were extracted in chloroform/methanol (2:1), then chloroform was evaporated [Grasselli et al. 2011]. Then, TG content was determined using the 'Triglycerides liquid' kit (Sentinel diagnostic, Milan, Italy). Spectrophotometric reading was performed with UV-VIS spectrophotometer.Values were normalized for the protein content. Data are expressed as percent TG content relative to controls.

2.10 ROS production and lipid peroxidation

The oxidation of the cell-permeant 2'-7' dichlorofluorescin diacetate (DCF-DA, Fluka, Germany) to 2'-7'dichlorofluorescein (DCF) allowed to quantify *in situ* the production of H_2O_2 and other ROS [Halliwell, Whiteman M , 2004]. Stock solution of DCF-DA (10 mM in DMSO) was prepared and stored at -20°C in the dark.. At the end of treatment cells were scraped and gently spun down (600xg for 10 min at 4°C). After washing, cells were loaded with 10 mM DCF-DA in PBS for 30 min at 37°C in the dark. Then, cells were centrifuged, suspended in PBS and the fluorescence was measured fluorometrically (lex=495 nm; lem=525 nm). All measurements were performed in a LS50B fluorimeter (Perkin Elmer, USA) at 25°C using a water-thermostated cuvette holder.

Lipid peroxidation was determined spectrophotometrically through the thiobarbituric acid reactive substances (TBARS) assay which is based on the reaction of malondialdehyde (MDA; 1,1,3,3-tetramethoxypropane) with thiobarbituric acid (TBA) [Iguchi et al. 1993]. Briefly, 1 vol. of cell suspension was incubated for 45 min at 95°C with 2 vol. of TBA solution (0.375% TBA, 15% trichloroacetic acid, 0.25 N HCl). Then, 1 vol. of N-butanol was added and the organic phase was read at 532 nm in a UV-VIS spectrophotometer at 25°C using Peltier-thermostated cuvette holder. The MDA level was expressed as pmol MDA/mL/mg protein.

2.11 Oil-Red O staining

Neutral lipids were visualized using the selective Oil-RedO (ORO) dye [Grasselli et al. 2010]. Briefly, after fixing in 4% paraformaldehyde, cells were washed three times with PBS, stained for 20 min with 0.3% ORO solution prepared from a stock 0.5% in isopropanol and diluted in water. After washing with distilled water, slides were examined by Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany).

2.12 Nitrite/Nitrate (NOx) Levels

NO production was measured by spectrophotometric measurement of the end products, nitrites and nitrates, using the Griess reaction [Green et al. 1982]. After treatments, nitrite accumulation (μmol $NaNO_2$/mg sample protein) was calculated against a standard curve of sodium nitrite ($NaNO_2$). All spectrophotometric analyses were carried out at 25°C recording absorbance at 540 nm.

2.13 Western blotting

Protein levels of NF-kB p65 were assessed by Western blot analysis. Briefly, the cellular pellet was suspended in 400 μL ice-cold Buffer A (20 mM Tris HCl pH 7.8, 50 mM KCl, 10 μg/mL Leupeptin, 0.1 mM Dithiothreitol-DTT, 1 mM phenylmethanesulfonyl fluoride-PMSF); then 400 μL Buffer B (Buffer A plus 1.2% Nonindet P40) was added. The suspension was vortex-mixed for 10 sec; after centrifugation the nuclear pellet was washed and resuspended in 100 μL Buffer B, mixed thoroughly in ice for 15 min and finally centrifuged. The supernatant containing the nuclear extracts was collected and the protein content was measured. About 30 μg proteins were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [LaemmLi, 1970]. Membrane was blocked in 5% fat-free milk/PBST (pH 7.4) and probed using rabbit anti-human NF-kB p65 antibody (SC-109; Santa Cruz Biotechnology, DBA, Milan, Italy). Membranes were incubated overnight at 4°C with primary antibody in PBST buffer (PBS with 0.1% Tween 20) washed and incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Sigma-Aldrich) in PBST for 1h at room temperature [Vergani et al. 2017]. Immune complexes were visualized using an enhanced chemiluminescence Western blotting analysis system (Bio-Rad ChemiDoc XRS System). Films were digitized and band

optical densities were quantified against the actin band using a computerized imaging system and expressed as Relative Optical Density (ROD, arbitrary units). ROD of each band was expressed as percentage respect to control.

2.14 Wound Healing assay

The migration of HECV was evaluated using the wound healing assay [Rodriguez et al. 2005]. The cells were seeded on 35×10 mm tissue culture dishes and incubated until confluence was reached, the cell monolayer was scraped with a p100 pipet tip making two crossing straight lines to create a ‘‘scratch’’. Then, five views on the cross were photographed by an inverted Olympus IX53 microscope (Olympus, Milan, Italy) and representative images were captured with a CCD UC30 camera and a digital image acquisition software (cellSens Entry). After scratching, medium was replaced with fresh medium in the absence or presence of each plant extract at 1.5 μg/ml concentration. Set of images were acquired at 0, 6 and 24 h. To determine the migration of HECV, the images were analysed using ImageJ free software (http://imagej.nih.gov/ij/). Percentage of the closed area was measured and compared with the value obtained before treatment. An increase of the percentage of closed area indicated the migration of cells. Data are means ± S.D. of at least three independent experiments.

2.15 Statistical analysis

Data are means ± S.D. of at least three independent experiments. Statistical analysis was performed using ANOVA with Tukey’s post-test (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

3.1 Phenol and flavonoid contents and radical scavenging activity of the extracts

Total phenol (TPC) and total flavonoid (TFC) contents were assessed in both extracts from *T. spicata* leaves by spectophotometric assays (Table 1). The ethanolic extract (TE) showed TPC higher than the aqueous one (TW) (250±6.9 *vs* 150±3.7 mgGAE/g of dry extract, respectively). Also the TFC was higher in TE than in TW (9.04±1.2 *vs* 4.21±1.06 mgQE/g of dry extract respectively)

The free radical scavenging capacity was evaluated by the DPPH assay. Both the extracts showed a concentration-dependent scavenging potential (data not shown). The antioxidant activity was similar for the two extracts as indicated by the similar IC50 values (24.5 µg/ml ±1.09 for TE, and 25.8 µg/ml ±1.28 for TW) (Table 1).

3.2 Chemical characterization of the aqueous and ethanolic extracts of T. spicata

The phenolic components of both extracts were identified using HPLC coupled with MS/MS in negative ionization mode; representative HPLC chromatograms are shown in Figure 1A supplementary data. We identified eighteen different polyphenols in TW, and thirteen in TE (Table 2). In both extracts, the most abundant classes of components were monoterpenoic phenols, polyphenolic acids, flavonoids and their derivatives. We observed some differences between them; rosmarinc acid (RA) was the most abundant PC in TW (38.60%), followed by salvalonic acid I (10.17%) and rutin (7.17 %), while carvacrol (CVL) was the most abundant PC in TE (36.84%), followed by thymusin (20.25%) and eriodictyol derivate (9.45%). Both extracts were rich in flavonoids such as apigenin, luteolin, quercetin and eriodictyol under form of aglycones or other derivatives (especially flavonoid glycosides).

We assessed the main volatile compounds in the extracts by GC-MS analysis; representative chromatograms are shown in Figure 1B supplemantary data. As summarized in Table 3, in the aqueous extract, only nine volatile compounds were identified including four phenols, with p-cresol 2,2 methylenebis(6-tert butyl) being the most abundant one (31.5%). The ethanolic extract contained a larger number (twenty-six) of volatile compounds with carvacrol being the most abundant component (68.8%).

3.3 T. spicata extracts counteract fat-dependent steatosis and oxidative stress in hepatic cells

To assess if the extracts affected viability of FaO cells, the MTT assay was carried on FaO cells treated for 24 h with increasing concentrations of TE or TW (0.15, 1.5 and 15 μg/ml) or with identical concentrations of pure carvacrol (CVL) and rosmarinic acid (RA) for comparison. In all cases, no significant changes in viability were observed (data not shown).

To mimic *in vitro* what is occurring in the liver during high fat feeding and/or obesity, FaO cells were overloaded of lipids by exposure to oleate/palmitate mixture (0.75 mM). Figure 1A shows that treatment with FAs induced a moderate steatosis (SS) quantified by the increase in the intracellular TG content (+155% compared to controls; $p \leq 0.0001$). When SS cells were treated for 24h with different concentrations of TW or TE a significant reduction in TG accumulation was observed, but the effect was not in dose dependent manner. In details, TW treatment at the doses of 0.15, 1.5 and 15 μg/ml led to a decrease in TG content of about -114%, -113%, and -104%, respectively ($p \leq 0.05$) compared to steatotic cells. At the same doses, TE led to a lower reduction in the TG content (-88%, -57% and – 67 % respectively; $p \leq 0.05$) compared to steatotic cells.

Cytosolic LDs were visualized and analysed by ORO staining and optical microscopy (Fig. 1B). Both the number and size of LDs increased markedly in steatotic cells compared to controls (from 1.3 to 5.3 LDs/cell, and from 0.9 to 2.65 □m of diameter). LD size and number changed upon incubation with extracts; LD size decreased to 1.52 □m with TW and to 1.65 □m with TE ($p \leq 0.001$, $p \leq 0.0001$) at the intermediate dose (1.5 □g/ml). The number of LDs/cell decreased significantly only with TE (4.3 LDs/cell for the intermediate dose; $p \leq 0.05$) (Fig.1C).

The oxidative stress was assessed by measuring lipid peroxidation by TBARS assay. After exposure to oleate/palmitate for 3h, the MDA level (Fig. 2) increased in steatotic cells (+86% compared to controls; $p \leq 0.0001$), and decreased when steatotic cells were exposed to both extracts for 24h. In details, TW treatment at the doses of 0.15, 1.5 and 15 μg/ml led to a decrease in MDA level with respect to steatotic cells of about -70%, -68% ($p \leq 0.01$) and -75% ($p \leq 0.001$), respectively. At the same doses, TE decreased the MDA level of about -110%, -110% and -102% ($p \leq 0.0001$). In control cells, MDA level was not affected by extracts (data not shown).

3.4 T. spicata extracts rescue the radical-dependent oxidative stress and dysfunction in endothelial cells

For experiments with HECV cells we chose the intermediate concentration of 1.5 □g/ml, after assessing that it did not affect cell viability (data not shown). HECV cells exposed to 20 μM H_2O_2 for 24 h showed a marked increase in MDA level (+43% with respect to controls; $p \leq 0.01$) (Fig. 3A). Treatment of H_2O_2-insulted cells with TW or TE **for 24** h partially counteracted this effect leading to a decrease in MDA level of about -37% ($p \leq 0.01$) for TW, and of -58% ($p \leq 0.0001$) for TE as compared to H_2O_2-insulted cells. The intracellular production of ROS was also visualized *in situ* by fluorescence microscopy of DCF-stained cells **compared** to controls, higher and diffuse DCF fluorescence was observed in H_2O_2-insulted cells, and it was reduced

after treatment with TW or TE for 24 h (Fig. 3B). When these changes were quantified by fluorimetric analysis we observed a significant DCF decrease, of similar extent, in H_2O_2-insulted cells treated with TW (-58%; $p \leq 0.05$) or with TE (-62%; $p \leq 0.05$) (Fig. 3C). Both MDA level and DCF signal were not affected by extracts in control cells.

The effect of extracts on NO production, a classical marker of inflammation, was assessed in HECV cells. In H_2O_2-insulted cells we observed a stimulation of NO release of +35% with respect of control ($p \leq 0.01$) (Fig. 4A). Both extracts were able to counteract this effect; TW and TE treatments decreased NO release compared to H_2O_2-insulted cells (about -57% and -43%; $p \leq 0.001$ and $p \leq 0.01$, respectively). NF-κB activation is a central mediator of inflammatory response in oxidative stress conditions. H_2O_2-insulted cells showed an increase in NF-κB p65 level with respect to control (+41%; $p \leq 0.01$); this increase was counteracted by both extracts (-44% for TW, and -77% for TE; with respect to stressed cells; $p \leq 0.001$ and $p \leq 0.0001$) (Fig. 4B).
We verified if the extracts were able to modulate the HECV migration ability by T-scratch assay. When the scratched confluent cell layer was incubated for 24 h in media containing TW or TE we observed an average acceleration of the wound repair compared to control (Fig. 5A). No significant differences in cell migration rate were observed at a short time after the scratch (6 h), whereas at a longer time (24 h), the control cells reduced the wound width of about 40% compared to time 0. Both extracts significantly stimulated cell migration resulting in a wound width smaller to that of controls at the same time, 32% for TE and 23% for TW (Fig. 5B).

DISCUSSION AND CONCLUSIONS

The Mediterranean diet consisting of many vegetal foods rich in phenolic compounds has known as one of the factors responsible for the reduced incidence of metabolic and cardiovascular (CVD) disorders in this area. Here, we have shown that extracts from leaves of *T. spicata*, an

aromatic plant of the east Mediterranean area, may counteract the excess hepatic accumulation of triglycerides (antisteatotic activity) and the oxidative stress (cytoprotection activity) in NAFLD models.

T. spicata is used as a healthy plant in Lebanon. The leaves are rich in phenolic compounds which received growing attention among nutritionists as possible therapy in the prevention of metabolic. In the present study, we tested two different extracts of *T. spicata* leaves, as the extraction solvent (water or ethanol) is important for the final composition. The water extraction is consistent with the traditional preparation of tea infusion of leaves, whereas the ethanol extraction is typical of pharmacological studies. As expected, the number of volatile species was larger in the ethanolic extract than in the aqueous one (twenty-six *vs* nine), indeed the abundancy of extracted molecules is one of the reasons to prefer alcohol extracts in pharmacology.

We, firstly, reported a detailed composition analysis of the extracts from *T. spicata* aerial parts which were prepared using water or ethanol as extraction solvents. Monoterpenic phenols and flavonoids were more abundant in the ethanolic than in the aqueous extract. According to previous reports [Dorman et al. 2004], rosmarinic acid was the most abundant PC in the aqueous extract. RA, a common polyphenol of the Lamiaceae family [Nunes et al. 2017], shows low toxicity and widespread biological properties, including antioxidant and anti-inflammatory effects [Jun et al. 2014], protective activity against stress–induced pathologies such as ischemia–reperfusion [Ramalho et al. 2014], and liver injuries induced by diabetes [Mushtaq et al. 2015] or drugs [Yang et al. 2013]. TW contained other components which may play synergic action with RA, such as the salvalonic acids (SAs), polyphenols with antioxidant, cardioprotective and anti-inflammatory potential [Tsai et al. 2010, Wang et al. 2010], rutin, vicinin 2 and other flavone derivatives, which are known for their health benefits [Hu et al. 2016, Zang et al. 2016].

Carvacrol was the most abundant component in the ethanolic extract; this monoterpene is a common component of essential oils from aromatic plants and spices, and it is known for its antibacterial, antifungal, anti-inflammatory, hepatoprotective and anti-carcinogenic activities [Erci et al. 2017, Friedman et al. **2014**]. TE was also rich in thymusin, a flavonoid with antiallergic [Sato and Tamura, 2015] and antidiabetic [Mercader et al. 2008] activities, and of *p-cymene-2,3-diol,* a semivolatile compound being characterized by an additional hydroxyl group with respect to carvacrol [Rainis and Ternes, 2014]. Moreover, *p-cymene-2,3-diol* was also present in dimeric form, the 3,4,3′,4′-tetrahydroxy-5,5′-diisopropyl-2,2′- dimethylbiphenyl, which was already found in leaves of *Thymus vulgaris* [Nakatani et al. 1989]. Based on these data, we might assume that the beneficial activity of TW could depend mainly on the rosmarinic acid, and that of TH on carvacrol, as they are the predominant agents in each extract.

In the liver, excess TG are stored in lipid droplets leading to steatosis, the hallmark of NAFLD. In order to explore and test potential drug candidates, we employed an experimental model consisting of rat hepatoma FaO cells exposed to a mixture of oleate/palmitate which represent a reliable *in vitro* model for hepatic steatosis widely employed in previous studies of our group [Vecchione et al. 2016, Vergani et al. 2017]. The moderate steatosis induced by FAs was reduced by both *T. spicata* extracts. However, the aqueous extract was most effective to counteract hepatic steatosis *in vitro* with respect to the ethanolic extract at all the tested doses (about -110% *vs* -60% of TG accumulation, respectively, compared to steatotic cells). The lipid-lowering activity of the extracts was depending on their action on lipid droplets: both the number and size of LDs increased markedly in steatotic cells compared to controls, and decreased of a similar value upon incubation with both extracts.

Excess fat accumulation in hepatic cells is accompanied by increased oxidative stress. As markers of oxidative stress we assessed both the intracellular ROS production, and the level of lipid peroxidation. Both these indices were significantly increased in steatotic cells compared to control hepatocytes. Incubation of steatotic cells with TW or TE led a significant reduction in the level of ROS and of lipid peroxidation. However, the ethanolic extract was most effective as anti-oxidant with respect to the aqueous extract at all the tested doses.

Endothelium is the blood–tissue interface; it is the first rate-limiting step in the utilization of long-chain FAs as fuels, and it is altered in NAFLD. Endothelial cells play regulatory functions through releasing various factors including ROS and nitric oxide. In the liver sinusoids, the endothelial cells act in hepatic fibrosis development by sustaining wound healing response and inflammation [Lalor et al. 2002]. HECV cells exposed to hydrogen peroxide can mimic what happens *in vivo* in atherosclerosis, as endothelial damage is typically observed in metabolic syndrome [Vergani et al. 2017]. The oxidative stress induced in HECV cells by exposure to H_2O_2 was partially counteracted by both extracts. The ROS production was visualized *in situ* by fluorescence microscopy and fluorimetric analysis and it resulted significantly decreased by both TW and TE. The decrease in lipid peroxidation was larger for TE (-58%) than for TW (37%) compared to H_2O_2-insulted cells. Also the increase in NO release, a key indicator of endotheliium inflammation and oxidative damage, was counteracted by both extracts (-57% for TW and -43% for TE). NF-κB is a master transcription factor in the activated by oxidative stress. We observed an increase in NF-κB p65 level in H_2O_2-insulted cells which was counteracted by both extracts but TE played a larger effect (-77%) compared to TW (-44%). The extracts were also assessed for their effects on endothelial cell migration. We observed that both extracts were able to accelerate the wound repair, however TE was more effective than TW (-32% *vs* -23% of

wound width, respectively). Taken together these data indicate that both the *T. spicata* extracts might ameliorate oxidative damage and inflammatory response in endothelial cells by inhibiting oxidative/nitrosative stress pathway and reducing the contents of intracellular NO and ROS. However, each extract showed some specificity in its antioxidant activity, with TE showing a higher antioxidant potential, likely depending on the panel of PC contained.

We can conclude that both extracts from *T. spicata* aerial parts are efficacious lipid-lowering and antioxidant agents for steatotic hepatocytes and activated endotheliocytes, representing reliable *in vitro* models of NAFLD. Interestingly, the aqueous extract was more effective as lipid-lowering and wound-repair agent, likely depending on its PC profile consisting of a high content of rosmarinic acid. By contrast, the ethanolic extract played a more marked anti-oxidant activity, both in hepatic and endothelial cells, possibly depending on its abundance of carvacrol. Moreover, the results suggest that the extracts and their single components, carvacrol and rosmarinic acid especially, act directly on hepatic and endothelial cells, although *in vivo* their effects may also be secondary to the anti-inflammatory/antioxidant responses in other tissues/organs.

In conclusion, the beneficial effects of the two extracts of *T. spicata* leave extracts which contain a different panel of compounds clearly indicate that this edible plant possesses a potential therapeutic benefit which explains its traditional use in Lebanon. Therefore, we suggest that this plant may have a great relevance as healthy and dietary supplement in the prevention and treatment of diseases involving free radicals and oxidants such as NFALD.

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Table 1: Total phenol content (TPC), total flavonoid content (TFC) and DPPH radical scavenging activity of *T. spicata* extracts. **Data are present in mean ±SD of experiments performed at least in quadruplicates**.

	TPC mgGAE/g dry extract	**TFC mgQE/g dry extract**	**IC50 (DPPH) µg/mL**
Ethanolic Extract (TE)	250±6.9	9.04±1.2	24.5±1.09
Aquesous Extract (TW)	150±3.7	4.21±1.06	28.5±1.28

Table 2: Phenolic compounds identified in *T. spicata* extracts by using HPLC-MS/MS in the negative ionization mode.

ETHANOLIC EXTRACT

a	RT (min)	Measured m/z	MS/MS fragments	Proposed compound	Percentage area (%)
1	14.1	593	575 503 473 383 353	Vicinin 2	2.56
2	14.5	303	285 177 125	Dihydroquercetin (taxifolin)	2.38
3	17.1	417	371 287 263	Eriodictyol derivate	9.45
4	18.5	609	301	Rutin	1.3
5	19.1	359	223 197 179 161 133	Rosmarinc acid	4.2
6	19.7	287	269 151 135 107	Eriodictyol	6.8
7	21.5	329	314	Thymusin	20.25
8	22.8	269	201 181 149	Luteolin	7.95
9	23	285	257 243 151	Apiginin	0.75
10	23.3	343	328 313 300 285	Unknown	1.24
11	24	165	149	p-cymene-2,3-diol	2.82
12	24.3	343	328 313	Cirsilineol	1.76
13	25.7	-	-	Carvacrol*	36.84
14	27.3	329	314 299 286 271	3,4,3′,4′-tetrahydroxy-5,5′-diisopropyl-2,2′-dimethylbiphenyl	1.64

[a] Compounds are listed according to their elution order in the reverse phase HPLC; * detected with standard authentic compound.

AQUEOUS EXTRACT

a	RT (min)	Measured m/z	MS/MS fragments	Proposed compound	Percentage area (%)
1	8.1	305	225	Gallocatechin	3.6
2	12.2	387	369 225 207 163	Tuberonic acid glucoside	0.5
3	14	593	575 503 473 383 353	Vicenin 2	3.76
4	15	637	461 351 285	Luteolin-O-diglucuronide	4.81
5	15.4	537	493 339	Salvalonic acid I	10.17
6	15.7	477	397 373 343 301	Quercetin-glucuronide	2.78
7	16.3	595	473 429 287	Eriodictyol-rutinoside	1.25
8	16.5	623	433 287	Luteolin-glucuronide-hexoside	1.73
9	17	717	537 519 475 365 339	Salvalonic acid E/B	5.85
10	17.4	461	285	Luteolin 7-O-glucuronide	4.16
11	17.6	593	285	Luteolin-O-rutioside	2.49
12	17.9	441	418 405 373 305 225 175	Unknown	2.05
13	18.1	521	359 179 161	Rosmarinic acid-glucoside	1.26
14	18.5	609	301	Rutin	7.17
15	19.1	359	223 197 179 161 133	Rosmarinic acid	38.6
16	19.7	549	387	Tuberonic acid derivate	4.37
17	19.8	607	559 427 299 284	Methyl kaempferol O-rutinoside	3.27
18	21.6	491	443 311 267	Salvanolic acid C	1.73
19	25.7	-	-	Carvacrol*	0.8

[a] Compounds are listed according to their elution order in the reverse phase HPLC; * detected with standard authentic compound.

Table 3: Chemical composition of volatile compounds of *T. Spicata* ethanolic extracts detected by GC-MS. Compounds are numbered in order of percentage.

Compounds of TE	**RT(min)**	**Percentage (%)**
Carvacrol	32.32	68.8
p-Cresol. 2,2-methylenebis(6-tert-butyl)	47.38	5.7
Pyrocatechol, 4-tert-butyl-	36.38	2.75
Methyl isosetearate	44.74	2.7
Palmitic acid methyl ester	42.8	2.6
Phenol,2,4-di-tert-butyl-	37.69	2.35
2-Hexadecanol	35.3	1.5
caryophyllene	35.87	1.5
Caryophyllene oxide	38.88	1.18
Dimethyloxybutane	4.01	1.15
Azulene, 7-isopropyl- 1,4 dimethyl-	40.63	1.1
Hexa hydro farnesol	33.14	0.9
p-Cymene	10.38	0.85
Cedrol	38.35	0.8
p cymen 7 ol	31.92	0.8
Thymol	31.79	0.7
Spathunlenol	38.78	0.68
Oleic Acid	20.52	0.4
Ocimene	11.72	0.35
5-Octadecenal	13.7	0.27
Z-11-Tetradecenoic acid	7.57	0.25
E-9-Tetradecenoic acid	17.76	0.2
Tran 13 octadeonic	34.75	0.18
cis-Vaccenic acid	38.57	0.18

6-Octadeoenoic acid	25.11	t
à-N-Normethadol	36.15	t

Compounds of TW	**RT(min)**	**Percentage (%)**
p-Crersol 2,2 methylenebis(6-tert butyl)	47.37	31.5
Palmitic acid methyl ester	42.8	10.8
Carvacrol	32.26	8.5
Azulene, 7-isopropyl- 1,4 dimethyl-	40.63	6.8
Ethyl iso-allocholate	44.74	6.6
Phenol,2,4-di-tert-butyl-	37.7	5.0
Hydroquinon, 2-6-di-tert-butyl	13.75	3
à-N-Normethadol	41.32	1.4
2-hexadecanol	31.11	1.2

FIGURE LEGENDS

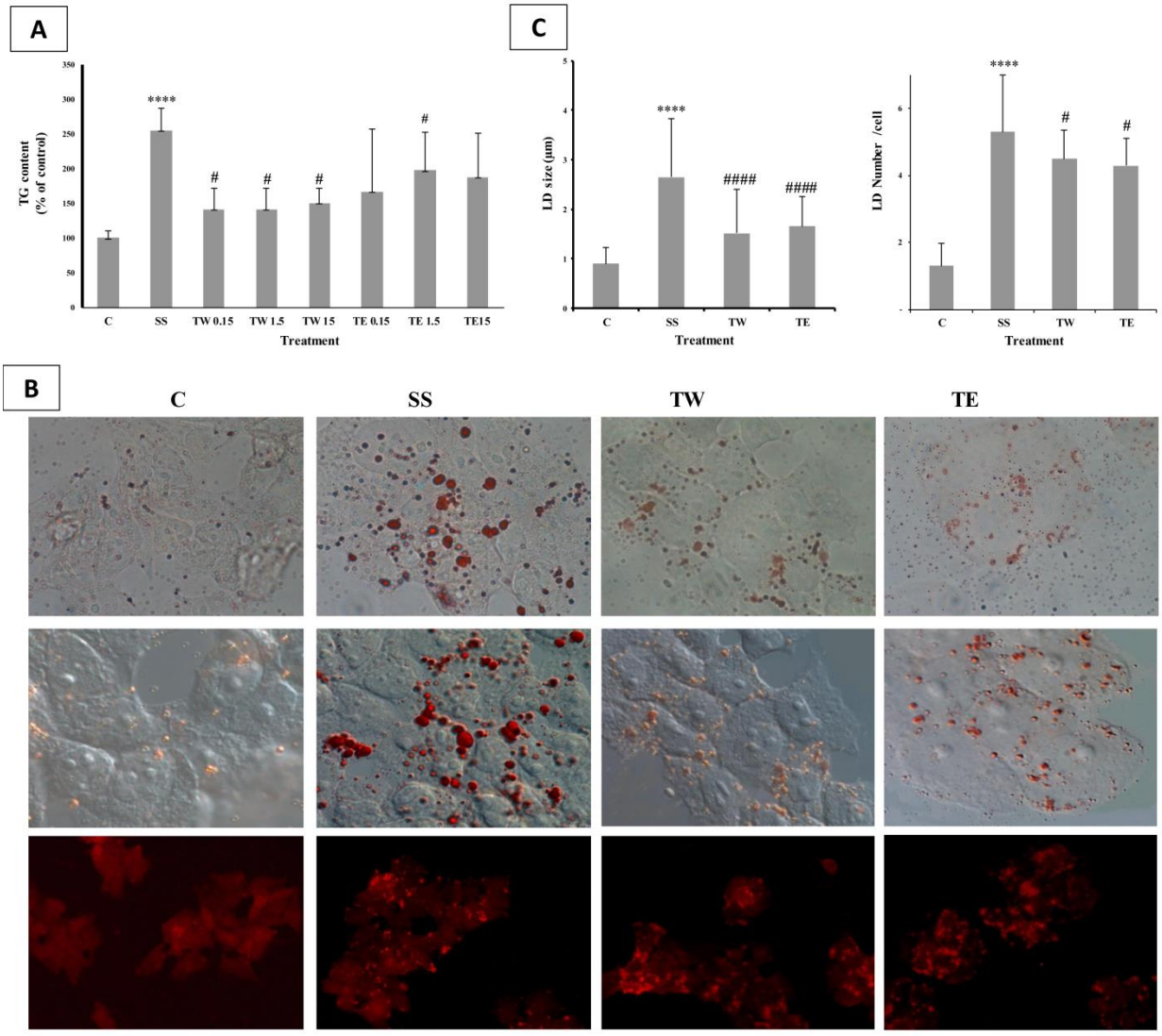

Figure 1: Effects of *T. spicata* extracts on lipid accumulation in hepatic cells.

(A) Triglyceride content was quantified by spectrophotometric assay in control and steatotic FaO cells incubated with FA mixture (SS) and then treated with *T. spicata* extracts (TE and TW) at different concentrations (0.15, 1.5 and 15 µg/ml) for 24h. Results are expressed as percent TG content relative to control and normalized for total proteins. **(B**) Neutral lipid accumulation was assessed i*n situ* in ORO-stained control and steatotic FaO cells (SS) and extracts-treated FaO cells (1.5 µg/ml). Images were acquired at Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany), magnification 100x; Bar: 100µm (**C)** Average size and number of Lipid Droplets per cell were calculated using ImageJ free software (http://imagej.nih.gov/ij/). Values

are mean ± S.D from a least three independent experiments. Significant differences are denoted by symbols: C *vs* SS ****p≤0.0001 and SS *vs* different concentration of extracts #p≤0.05**.**

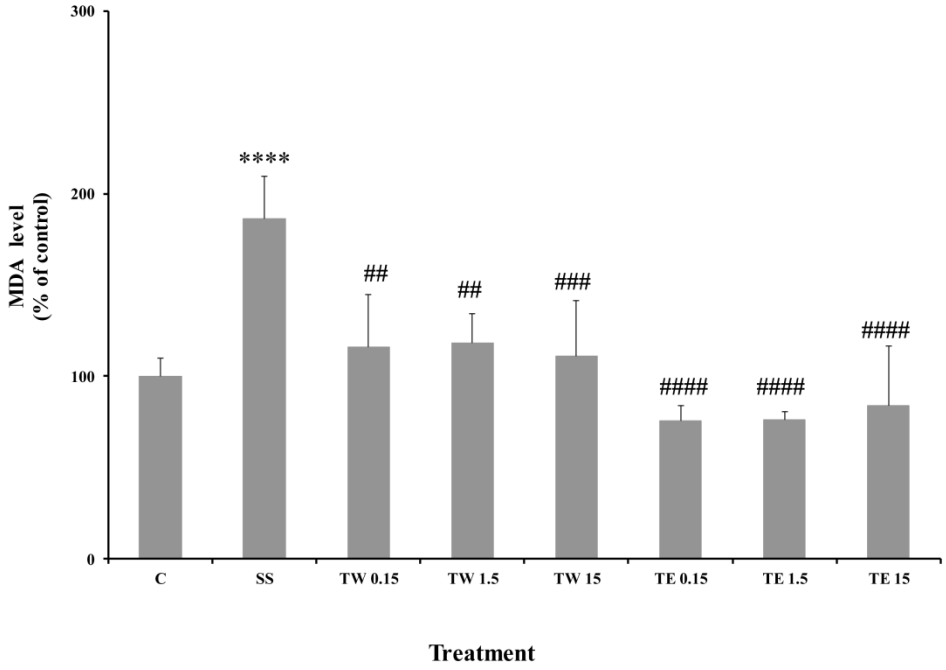

Figure 2: **Effects of *T. spicata* extracts on lipid peroxidation in hepatic cells**

(A) Intracellular MDA level was quantified by TBARS assay as pmol MDA/mL x mg of sample protein. Values are reported as % of control and are mean ± S.D from a least three independent experiments. Significant differences are denoted by symbols: C *vs* SS ****p≤0.0001 and SS *vs* different concentration of extracts #### p≤0.0001, ### p≤0.001, ##p≤0.01, #p≤0.05.

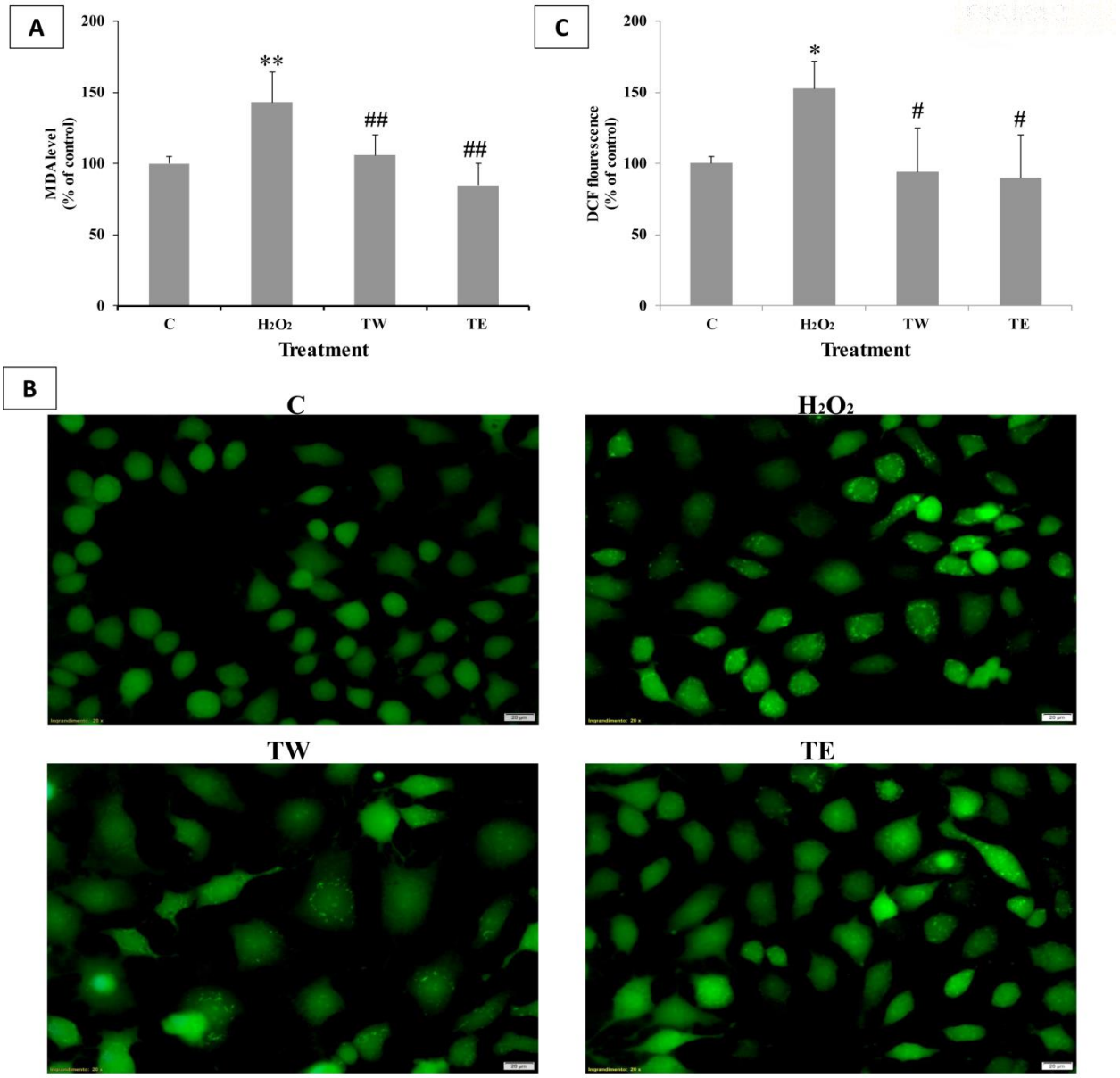

Figure 3: Effects of *T. spicata* extracts on lipid peroxidation and ROS production in endothelial cells.

HECV cells treated with H_2O_2 (20µM) for 24h to induce oxidative stress were then treated with both extracts at the intermediate concentration (1.5 µg/ml) for 24h. **(A)** Intracellular MDA level was quantified by TBARS assay; **(B)** The intracellular ROS production was visualized *in situ* by fluorescence microscopy of DCF-stained cells. Images were acquired at Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany), magnification 20x (Bar: 20µm); **(C)** DCF fluorescence was quantified by spectrofluorimeter assay of DCF-stained cells. Values are mean ± S.D from a least three independent experiments. Significant differences are denoted by symbols: C *vs* H_2O_2 insulted cells **p≤0.01, *p≤0.05 and H_2O_2 insulted cells *vs* different concentration of extracts, ### p≤0.001, ##p≤0.01, #p≤0.05.

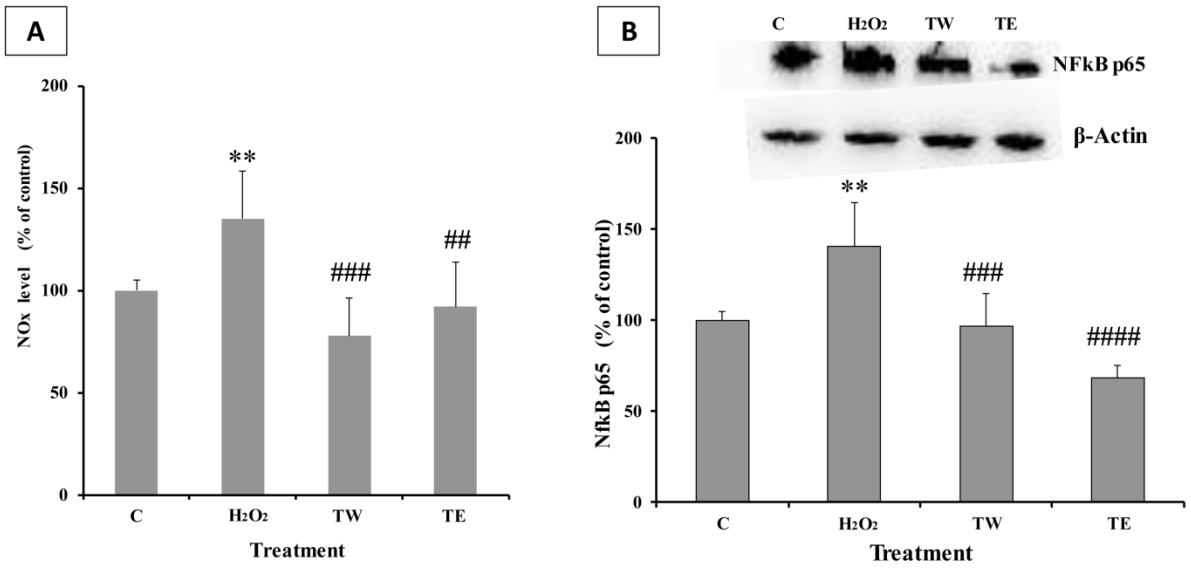

Figure 4: Effect of *T. spicata* extracts on oxidative stress-induced dysfunction in endothelial cells

HECV cells treated with H_2O_2 (20µM) for 24h were treated with both extracts at the intermediate concentration (1.5 µg/ml) for 24h **(A)** Nitric oxide production was quantified in the medium of HECV cells as µmol $NaNO_2$/mg sample protein by Griess reaction. Values are expressed as % of control **(B)** Densitometric analysis of nuclear NF-kB/p65 was evaluated by Western blotting; β-actin was the housekeeping gene for normalization; data are expressed as percentage values with respect to controls

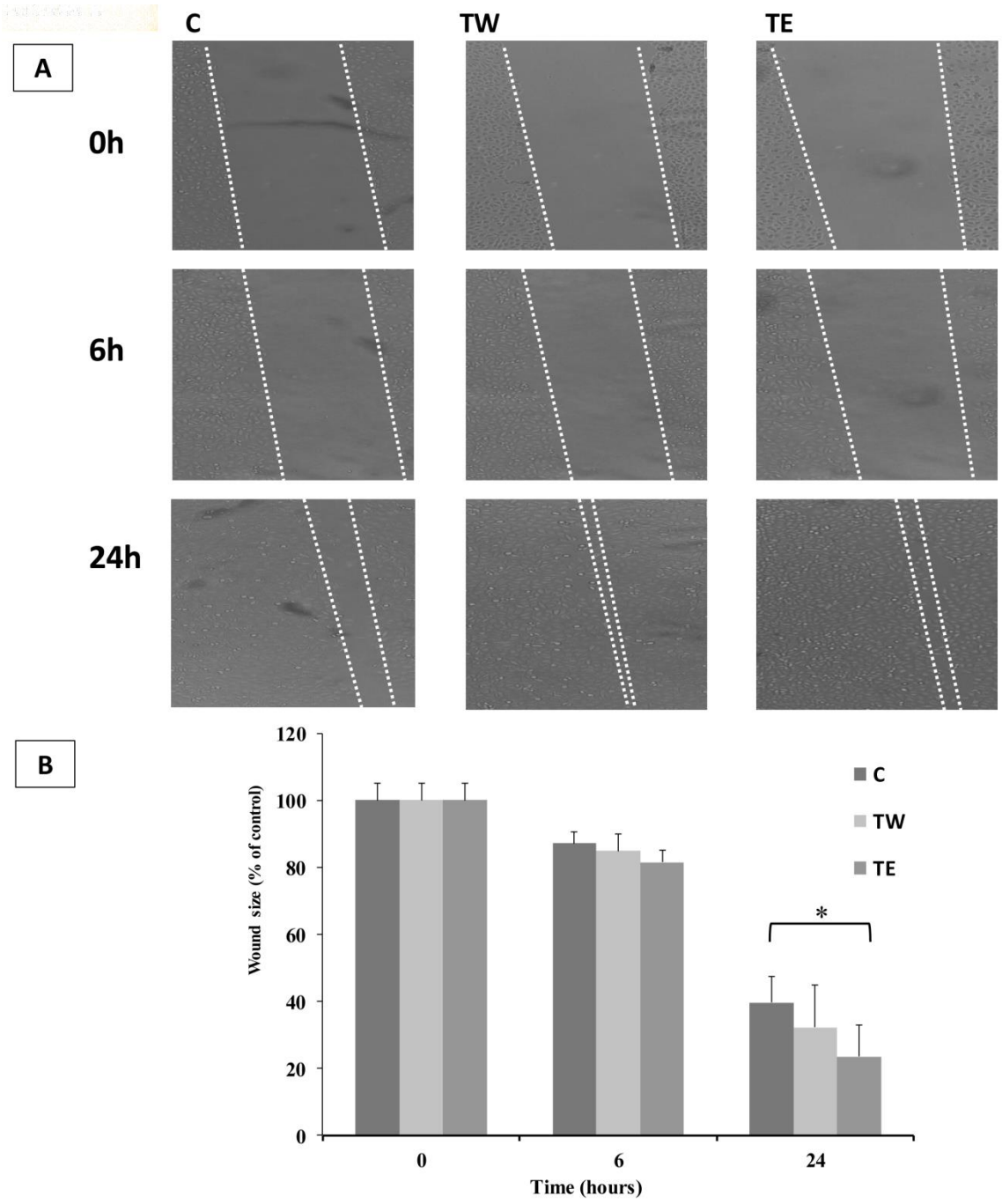

Figure 5: Effect of T. spicata extracts endothelial cell migration

HECV cells were treated with both extracts at 1.5 µg/ml, and subjected to wound healing assay as described in Materials and Methods. Images were acquired at 0, 6 and 24 h from the beginning of the assay. (A) The dotted lines define the areas lacking cells. (B) Graphs representing the percentage of the closed area as compared to time=0. T scratch assay representative images are also shown. Values are mean ± S.D from at least three independent experiments. Significant differences are denoted by symbols C *vs* TE treatment *$p \leq 0.05$.

➢ CHAPTER 2.

Antitumor activity of ethanolic extract from *Thymbra spicata L.* aerial parts: effects on cell viability and proliferation, apoptosis induction, STAT3 and NFkB signaling

This work is under review in the *Nutrition and Cancer: An International Journal*

ABSTRACT

Thyme-like plants including *Thymbra spicata L*. are widely used as food and folk medicinal remedy in the Mediterranean area. The present study aimed to explore the *in vitro* antitumor potential of polyphenol-enriched extracts from aerial parts of *T. spicata*. The ethanolic extract significantly inhibited proliferation of different human tumor cell lines, without significant effects on non-neoplastic cells. A deeper investigation of the molecular mechanism sustaining the *in vitro* antitumor activity of the extract was carried on the human breast cancer cells MCF-7 in comparison with the normal breast cells MCF-10A. The effects on MCF-7 cells were associated to: *(i)* production of reactive oxygen species (ROS) and release of nitric oxide; *(ii)* apoptosis induction; *(iii)* reduction in STAT3 and NF-kB phosphorylation. The ethanolic extract from *T. spicata* leaves might represent a novel therapeutic tool in combination with conventional chemotherapy to reduce the adverse side effects and drug resistance.

INTRODUCTION

Despite early detection, and progress in screening and therapies, cancer is the second cause of death in the world [World Health Organization (WHO) |Cancer, 2019]. Moreover, current chemotherapy strategies are often accompanied by numerous side effects depending on cytotoxicity of the drugs on normal tissues [Liu et al., 2015]. Therefore, there is a growing interest in the identification of new bioactive components with antiproliferatve effects on tumor cells and negligible effects on non-tumor cells which could be employed to improve the classical therapeutic approaches [Greenwell and Rahman, 2015].

Phytochemicals are compounds isolated from foods, beverages and plants which exert many biological activities and find applications in pharmaceutical industry. Many phytochemicals have gained attention as possible antitumor compounds [Cragg and Newman, 2000; Chen, Myracle, Wallig & Jeffery, 2016; El Hasasna et al., 2016]; indeed, they may inhibit cell proliferation, induce apoptosis, modify the ubiquitin-proteasome system [Fulda, 2010; Golonko et al., 2019]. Phenolic compounds (PC) are phytochemicals with at least one hydroxyl group attached to one or more aromatic rings in its chemical structure. PC may exert either cytoprotective or, alternatively, cytotoxic activity depending on the signaling pathways they affect [Lewandowska et al., 2016; Pan et al., 2018]. While the beneficial effects mainly depend on their antioxidant potential [Russo, Tedesco, Spagnuolo & Russo, 2017], their cytotoxicity could result from excess generation of reactive oxygen species (ROS) [Mileo and Miccadei, 2016]. The most common phytochemicals being employed in cancer therapy include: alkaloids such as vinblastine and vincristine from *Catharan roseus* for treatment of lymphomas, leukemias and solid tumors; terpenes such as Taxol from *Taxus brevifolia* Nutt. for treatment of breast, ovarian, and lung cancers; quinolones such as camptothecin from *Camptotheca acuminate* for treatment of leukemia [Gordaliza, 2007; Orlikova et al., 2014].

The Lamiaceae family includes many aromatics plants that are rich in phenolic compounds [Xu et al. 2018]. Within this family, thyme-like plants find traditional applications in the Middle East area to treat/prevent many diseases [Abu Rabia, 2015; Jaradat et al. 2016; Ahmad et al., 2017; Abu-Darwish and Efferth, 2018]. In Lebanon, thyme-like plants compose a popular mixture, called "zaatar", that is employed as food in salads and spices [Al Hafi et al. 2017; Dbaibo,

Bashour, Hamadeh & Toufeili, 2019]. Moreover, "zaatar" is used to alleviate cramps, muscle pains, hyperglycemia and inflammation [Hajhashemi, Sadraei, Ghannadi & Mohseni, 2000; Cam et al. 2019]. The main components of "zaatar" are *Thymbra capitata* [Delgado-Adámez et al. 2017; Abdallah et al. 2018] and *Thymbra spicata* [Eruygur, Çetin, Ataşn & Çevik, 2017]; studies showed that these plants exert antiproliferative effects *in vitro* [Loizzo et al. 2007; Srancikova, Horvathova & Kozics, 2013; Hassan et al. 2018; Kubatka et al., 2019].

Leaves of *T. spicata* contain large amounts of (poly)phenolic compounds such as phenolic acids (rosmarinic acid), phenolic monoterpenes (carvacrol) and flavonoids [Dorman et al., 2004; Khalil et al., 2019]. Phenolic monoterpenes are known for their antitumor, pro-apoptotic, and antiproliferative effects against many cancer cells [Arunasree, 2010; Chen et al., 2015; Llana-Ruiz-Cabello et al., 2015; Khan et al., 2018]. Carvacrol (5-isopropyl-2-methylphenol) is very abundant in both essential oils and organic extracts of *T. spicata* [Al Hafi et al., 2017; Khalil et al., 2019].

Here, we assessed the possible *in vitro* antitumor activity of polyphenolic extracts from *T. spicata* aerial parts by using different cancer cell lines being representative of the most common human cancers. As possible molecular mechanisms sustaining the extract activity we investigated proliferation inhibition and apoptosis induction, stimulation of ROS and NO production, inhibition of STAT3 and NF-kB activation.

MATERIAL & METHODS

2.1 Plant collection and extraction

Aerial parts of *Thymbra spicata* L. were collected from flowering plants growing in *"Maarake"* in south Lebanon. Voucher specimen (L1.125/1) was authenticated by Prof. George Tohme, (CNRS, Beirut, Lebanon) and was kept in the Herbarium of the botanical department, Lebanese University, Beirut, Lebanon. For aqueous extraction, 15 grams of dried materials were extracted with double-distilled water (ddH_2O) for 3 hours at room temperature, and then for 10 min at 60°C. For ethanolic extraction, 25 grams of dried aerial parts were extracted by maceration with 100% ethanol for 24h. After evaporation in a Rotavac vario power unit (Heidolph Instruments,

Schwabach, Germany), samples were freeze-dried in Alpha 1-4 LD plus lyophilizer (CHRIST, Osterode am Harz, Germany) and stored at 4°C.

2.2 HPLC analysis

High-performance liquid chromatography was performed on an Agilent 1100 HPLC-MSD Ion Trap XCT system (Agilent Technologies, Palo Alto, CA, USA). Separation was performed on a Jupiter C18 (1 mm×150 mm, 3.5 μm) column (Phenomenex, Torrance, USA) at the flow of 0.05 mL/min at 30°C using eluent A (water) and eluent B (MeOH) acidified with 0.1% formic acid. The gradient employed was: 5 min with 15% of eluent B which was increased to 100% in 35 min, decreased to 80% in 5 min, and finally hold at 100% for 5 min. We identified the major components of each extract by comparing their retention times to those of the authentic standards. For the analyses stock solutions (0.5 mg/mL) of the extracts and standards were prepared in 80% aqueous methanol. The injection volume was 8 μL. The concentration range for calibration was 0.001-0.5 mg/mL for both Carvacrol (CVL) and Rosmarinic acid (RA) [Khalil et al., 2019].

2.3 Cell Lines and Culture Conditions

Jurkat (acute T cell leukemia cells), HeLa (human cervix carcinoma cells), A549 (non-small cell lung cancer cells), MCF-7 (human breast adenocarcinoma cells), PC-3 (human prostate adenocarcinoma cells) and MCF-10A (human breast epithelial cell spontaneously immortalized) were supplied by Anti-cancer Therapeutic Approaches Group (ATAC, Lebanese University, Beirut, Lebanon). Tumor cells were routinely maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM) or in RPMI-1640 (Sigma Aldrich, Beirut, Lebanon supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM glutamine, 1% P/S at 37°C in a humidified incubator containing 5% CO_2.

2.4 Cell viability assay

The possible cytotoxicity of *T. spicata* extracts was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method according to Hussain et al, 1993. Stock solution (50 mg/mL) of both the ethanolic extract and the pure phenolic compounds (CVL and RA) were prepared in dimethyl sulfoxide (DMSO), and in sterile distilled water for the aqueous extract. For

analysis, cells were seeded in 96-well plate (10^4 cells per well), in complete medium. After 24 h, cells were treated with increasing concentrations (0, 25, 50, 75, 100, and 150 μg/mL) of each extract for 24 hours. At the indicated times, 20 μL of MTT reagent (5.0 mg/mL) was added and incubated at 37°C for 3 h. Then, the unreacted MTT dye was removed by aspiration and 100 μL of acidified isopropanol was added to solubilize purple formazan crystals within metabolically active cells. The absorbance of sample was recorded at 570 nm by multidetection microplate reader (Biotek Instrument, Winooski, VT, USA). Data are expressed as percentage of cell viability with respect to control. The IC_{50} value (concentration that causes 50% growth inhibition) was estimated as that leading to 50% absorbance decrease as compared to control.

The inhibitory rate was calculated by the following formula:

$$\text{Inhibitory rate (\%)} = (1- At/Ac) \times 100$$

Where *At* and *Ac* were the absorbance values of the treatment and control wells, respectively.

2.5 Micronucleus test

The number of micronuclei in MCF-7 cells exposed to extracts or single compounds were assessed following Valovicova et al. (2009). About 5×10^4 cells were seeded on coverslips in 6-wells plates. After 24 h cells were treated with either extracts or carvacrol. After 24 hours of treatment cells were, washed with PBS and fixed with 4% paraformaldehyde for 20 min. Then, cells were stained with DAPI (1 μg/mL in PBS) for 10 min. Coverslips were mounted onto the slides using mounting media and observed under a Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany).

2.6 Colony formation assay

Colony formation assay was performed to assess cell proliferation (Crowley, Christensen, Waterhouse, 2016). Briefly, MCF-7 cells were seeded in 6-well plates (250 cells/well) and incubated for 24 h at 37℃. Then, cells were treated with either extracts or single compounds. After incubation for 24 h, medium was changed with complete medium and cells were grown for 7 days until colonies appeared. Then, cells were washed with PBS and fixed in 4% paraformaldehyde for 20 min. After washing cells were stained with a 0.1% crystal violet

solution for 20 min, washed with distilled water and air-dried at room temperature. The counting of colony number was carried on by using an inverted Olympus IX53 microscope (Olympus, Milan, Italy). Images were captured with a CCD UC30 camera and a digital image acquisition software (cellSens Entry).

2.7 Transwell migration assay

MCF-7 cells (5×10^4/well) were seeded in an insert system (Corning, NY, USA), left to grow to 70-80% confluence, and then cells were starved for 12 h. Cells were incubated for 10 hours in with non-cytotoxic concentrations of either extracts or Carvacrol in starvation medium. After washing in PBS, cells were fixed with 4% paraformaldehyde for 20 min and then with 100% methanol for 10 min. Finally, cells were stained with crystal violet staining solution (1% in methanol) for 10 min, washed with PBS and air-dried. Cells from five random fields were counted [Justus, Leffler, Ruiz-Echevarria & Yang, 2014] under an inverted Olympus IX53 microscope equipped with CCD camera.

2.8 ROS production assay

2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA; Molecular probe) was employed to assess ROS generation [Halliwell and Whiteman, 2004]. Briefly, MCF-7 cells were seeded in 6 well plate (2×10^5 cells/mL) and incubated overnight. After treatments cells were disassociated by trypsinization, suspended in PBS with 10 μM H2DCFDA for 30 min at 37°C. Then, cells were spinned and resuspended in PBS and the fluorescence (λex=495 nm; λem=525 nm) was measured using a LS50B fluorimeter (Perkin Elmer, USA) at 25°C with a thermostated cuvette.

2.9 Quantification of Nitrite/Nitrate Levels

The amount of NOx (nitrites and nitrates) level was measured by spectrophotometric measurement using the Griess reaction (Green et al. 1982). MCF-7 cells were seeded in a 6 well plate (2×10^5 cells/mL) and incubated overnight. After 24 h of treatment with TE or CVL, nitrite/nitrate level) in the medium was calculated using sodium nitrite ($NaNO_2$) as a standard curve. Spectrophotometric analyses were performed at 540 nm using U-2900 UV-Vis spectrophotometer 200 V (Hitachi High-Technologies, Japan).

2.10 Western Blotting analysis

MCF-7 cells (1×10^6) were seeded in 10 cm petri dishes and grown for 24 h. At the end of treatments, cells, scraped, and lysed for 15 min on ice in RIPA Buffer containing protease inhibitor cocktail (Sigma Aldrich, Beirut, Lebanon) and phosphatase inhibitor cocktail (bioWORLD, Dublin, USA). Then, cell lysates were centrifuged at 14,000 rpm for 20 min at 4°C. Protein concentration was calculated by Lowry protein assay kit (BioRad, Beirut, Lebanon). 30-40 μg proteins were resolved into 10–15% SDS-PAGE and then transferred to nitrocellulose membranes (Biorad, Beirut, Lebanon). After transfer, membranes were blocked with 5% non-fat milk in PBST for 1 h at room temperature. Anti-hSTAT3, anti-Phos-hSTAT3, anti-phos-RelANFkB p65, , anti-hBcl2 and anti-hGAPDH antibodies was purchased from R&D systems (Minneapolis, USA). Membranes were incubated with primary antibodies overnight in blocking buffer at 4 °C. Horseradish peroxidase-conjugated anti-IgG was used as secondary antibody for 1 hour at room temperature. Chemiluminescence system (Bio-Rad ChemiDoc XRS System) was used to visualized the immune complexes and then the band optical densities were quantified against the GAPDH band using ImageJ free software (http://imagej.nih.gov/ij/).

2.11 Wound Healing Assay

MCF-7 cells were plated in 6-well plates and left to grow at 37℃. After confluency, the monolayers cells were wounded with a sterile pipette tip. Cells were washed and incubated with fresh medium (controls) and treatments. Wounded areas were photographed after 0, 24 and 48 hours of incubation by an inverted Olympus IX53 microscope (Olympus, Milan, Italy) and representative images were captured with a CCD UC30 camera and a digital image acquisition software (cellSens Entry). Scratch width was measured using ImageJ free software (http://imagej.nih.gov/ij/), for both control and treated cells, and normalized with respect to their respective values at 0 h. Migration rate was measured and compared with the value obtained for control untreated cells. A decrease in the wound area reduction is a sign of a reduction in cell migration ability. Data are means ± S.D. of at least three independent experiments [Rodriguez, Wu & Guan, 2005].

2.12 Apoptosis Detection by FACS

Cell apoptosis was determined using the FITC Annexin V Apoptosis Detection Kit (Becton Dickinson Canto II, BD Biosciences, San Jose, USA). After treatments, MCF-7cells were

harvested, scraped, and stained with Annexin V-FITC for 15 min at room temperature in the dark. After washing, 7-amino-actinomycin d (7-AAD) was added to cells in order to discriminate between early and tardive apoptotic cells. Measurements were performed using a FACS instrument (Becton Dickinson Canto II, BD Biosciences). Apoptotic fractions (early and late apoptosis) was visualized and quantified by constructing a dot plot using the BD FACSDiva software.

2.13 Statistical Analysis

Statistical analysis was performed using GraphPad Software, Inc., San Diego, CA, USA. Data are reported as means ± S.D of at least three independent experiments. The data were analyzed via one-way ANOVA with Tukey's post-test.

RESULTS

3.1 Chemical composition of the extracts of *T. spicata*

Aerial parts of *T. spicata* contain a large number of phenolic compounds as determined by HPLC-MS analysis. In the aqueous extract (TW), rosmarinc acid (RA) was the most abundant component (38.60%), followed by salvalonic acid I (10.17%) and rutin (7.17 %). In the ethanolic extract (TE), carvacrol (CVL) was the most abundant component (36.84%), followed by thymusin (20.25%) and eriodictyol derivate (9.45%) [Khalil et al., 2019]. In each extract, the concentrations of both carvacrol and rosmarinc acid were exactly quantified using the calibration curves obtained with authentic standards (Table 1). CVL was more abundant in TE than in TW (257.69±10.6 mg/g extract and 8.58±1.13 mg/g, respectively), whereas RA was ten folds more abundant in TW than in TE (488.3±7.39 mg/g extract and 42.8 ±4.76 mg/g extract, respectively). Representative HPLC chromatograms of the two extracts and of the authentic standards (rosmarinic acid and carvacrol) are reported in Figure 1.

3.2 Cytotoxicity of the aqueous and ethanolic extracts

The *in vitro* cytotoxicity of *T. spicata* extracts was evaluated on a panel of cancer cell lines including Jurkat, HeLa, A549, PC-3 and MCF-7 cells, and on the normal breast epithelial cell

line MCF-10A. Cells were treated for 24 h with increasing concentrations of each extract (0, 25, 50, 75, 100, and 150 μg/mL) or to vehicle alone (DMSO), and the cell viability was assessed by MTT assay. TE reduced cell viability of all cancer cell lines under analysis in a concentration-dependent manner (Fig. 2A), whereas TW had no significant cytotoxic effect on all the tested cell lines. When the IC50 value of TE was determined for each tumor cell line, we observed the greatest sensitivity in HeLa cells (IC50 = 45±5.4 μg/mL), and the lowest one in A549 cells (IC50 = 129±6.4 μg/mL), and intermediates values for the other cell lines. On the other hand, the vehicle alone did not alter cell viability significantly (data not shown). The non-neoplastic human breast cell line MCF-10A did not show any reduction in cell viability upon TW treatment, but also TE played only a slight cytotoxicity on normal cells (Fig. 2A).

For further investigations we focused on the MCF-7 cell line which shows an intermediate IC50 value (80 μg/mL). This is an estrogen receptor (ER)-positive breast cancer cell line. The non-neoplastic breast cells MCF-10A were used as comparison. On both breast cell lines we tested the effects of both pure carvacrol and rosmarinic acid as representative of TE and TW, respectively. While carvacrol alone led to a dose-dependent decline in both MCF-7 and MCF-10A cell viability, rosmarinic acid alone had no significant effect and this confirms the results obtained with the extracts (Fig.2B). Interestingly, pure carvacrol was less cytotoxic than TE for MCF-7 tumor cells (IC50 >100 μg/mL for CVL), but it was more cytotoxic for the normal cell line MCF-10A (inhibition of -46% for CVL vs -29% for TE, at high dose, 100 μg/mL).

3.3 Effects of the ethanolic extract on proliferation and migration of MCF-7 cells

In vitro colony formation assay was employed to evaluate the ability of cells to form colonies. MCF-7 cells were treated for 24 h with TE or CVL (at doses of 50 and 100 μg/mL), then left to grow for 7 days up to form visible colonies. As shown in Figure 3A, there was a reduction in the number of colonies upon treatments, which is a sign of massive cell death. In quantitative terms (Fig.3B), MCF-7 cells treated with the highest dose of TE (100 μg/mL) showed a marked decrease in the number of colonies compared to untreated cells (about -66%, $p<0.0001$), and this effect was higher than that observed with the same dose of pure CVL (about -39%; $p<0.01$). The results further demonstrated that TE is more effective than carvacrol on reducing the colony formation of MCF-7 cells.

The ability of MCF-7 cells to recover the proliferative capability after treatment removal was also assessed. After exposure to the highest dose of TE or CVL (100 µg/mL) for 24 h, cells were washed in PBS and incubated in fresh medium without treatment for 48 h, then cell viability was assessed. MCF-7 cells treated with TE (Fig. 3C) did not show any proliferative recovery, but kept on dying (-13% of viable cells after drug removal), while a moderate and non-significant recovery in cell viability was observed with pure CVL (+9% after drug removal). The results indicate that TE exerts an almost irreversible anti-proliferative effect on MCF-7 cancer cells, more marked than CVL.

Cell migration plays a crucial role in tumor metastasis, and the wound-healing assay was used to investigate the effects of TE and CVL at not cytotoxic concentrations (25 µg/mL). As shown in the representative figure 4A, in control MCF-7 cells the wound was gradually filled by migrating cells. No significant differences in the wound size could be appreciated at 24 h after scratching. At 48 h, cells treated with TE or CVL showed a slower migration rate compared to untreated cells. In details, at 48 h the control cells reduced the wound width of about 54% compared to time 0. Both TE and CVL reduced cell migration resulting in a wound width larger to that of controls at the same time (about 41% for TE compared to time 0; $p<0.5$) (Fig. 4B).

The ability of both TE and its main component carvacrol to affect the migration of MCF-7 cells was confirmed by the insert trans-well assay. The number of cells that migrated from the upper to the lower chamber was reduced significantly by TE (-27% compared to the control; $p<0.5$), but not by CVL (Fig. 4C-D).

3.4 Pro-apoptotic activity of the ethanolic extract

Optical microscopy analysis of MCF-7 cells showed that both TE and carvacrol modified the cell morphology after 24 h of treatment (Fig. 5A). While untreated cells (control) showed typical epithelial morphology and were well spread to the substrate, both TE and carvacrol (50 µg/mL) reduced the cell size leading to cell shrinking. At the highest dose (100 µg/mL), CVL treatment resulted in a subpopulation (about 60%) with a round shape typical of an early apoptosis-like phenotype, whereas TE treatment was more damaging leading to a big number of cells (about 80%) with plasma membrane disruption, cytoplasm translucency, apoptotic bodies being characteristic of an advanced apoptosis-like phenotype [Balvan et al., 2015].

The pro-apoptotic effects of TE and carvacrol were confirmed by other assays. The expression of Bcl-2, a general marker for apoptosis, was assessed by Western blot analysis (Fig. 5B). Also in this case, TE was more effective than carvacrol on reducing the level of Bcl-2 protein with respect (-31% and -14%; $p<0.0001$ and $p<0.5$, respectively) compared to untreated cells (Fig. 5B). Moreover, the apoptotic fraction was quantified by Annexin V/7-AAD FACS analysis (Fig. 5C-D). Compared to control, carvacrol (50 and 100 μg/mL) increased the fraction of both early apoptotic cells (Annexin V+/7-AAD-) up to 11% and 59%, respectively, and of late apoptotic cells (Annexin V+/7-AAD+) up to 11% and 27%, respectively. More marked was the effect of TE, which at the lowest dose (50 μg/mL) increased the fraction of early (+35%) and late (+12%) apoptotic cells, while at the highest dose (100 μg/mL) led to almost total late apoptosis population (+84%). At last, the micronucleus test confirmed the TE was more effective than carvacrol as apoptosis inducer. Cells undergoing apoptotic process showed highly-condensed chromatin which at later stages became fragmented leading to classical apoptotic bodies. Higher levels of DNA fragmentation and number of apoptotic bodies could be visualized in cells exposed to TE than in cells exposed to carvacrol at the dose of 50 and 100 μg/mL (Fig. 5E).

3.5 Ethanolic extract activity is mediated by ROS and NOx production

ROS generation was detected by fluorimetric analysis using the probe DCFH-DA (Fig. 6A). We observed not significant ROS generation in MCF-7 cells treated with 50 μg/mL of TE or carvacrol compared to control. Marked changes in ROS levels were observed with the highest doses of both compounds (+60% for CVL, and +70% for TE; $p<0.01$). The production of ROS was confirmed *in situ* by fluorescence microscopy of DCF-stained cells (Fig. 6C). In line with fluorimetric analysis, higher and more diffusive DCF fluorescence was observed in both CVL- and TE-treated cells when compared to untreated counterparts.

TE was also able to stimulate markedly the release of NO in MCF-7 cells (Fig. 6B). In fact, while carvacrol led to a non-significant increase in NO production, TE increased the NO level up to +73 % ($p<0.5$) and to +245% ($p<0.0001$) at doses of 50 μg/mL and 100 μg/mL, respectively, compared to control.

3.7 Etanolic extract activity is mediated by STAT3 and NFκB signaling

STAT3 activation is a mediator of tumorigenesis, and the level of pSTAT3 was assessed by Western blot analysis. TE markedly reduced the phosphorylation of STAT3 (-8% for TE 50 μg/mL, and -38.5%, for TE 100 μg/mL, $p<0.001$) with respect to untreated cells (Fig. 6D). TE was more potent than carvacrol which led to a non-significant reduction of pSTAT3 (-10%) only at the highest dose (100 μg/mL).

NF-kB signaling regulates the expression of many genes involved in tumor cell survival and proliferation. By Western blot analysis, we found that TE significantly reduces p65 phosphorylation compared to untreated cells (-9% for TE 50 μg/mL, and -38% for TE 100 μg/mL; $p<0.001$). By contrast, carvacrol at the highest dose (100 μg/mL) led to a slight and non-significant reduction of p65 phosphorylation (-13%) (Fig. 6E).

DISCUSSION

In the last decades, the research of novel antitumor agents of vegetal food has gained increasing attention in the attempt to develop effective and safer drugs [Srivastava and Srivastava, 2019]. In this study, we tested the antitumor potential of *T. spicata*, an aromatic plant widely employed as food in Lebanon with the name of "zaatar". Aerial parts were extracted using water (TW) or ethanol (TE) as extraction solvent. We demonstrated that only the ethanolic extract reduced significantly both viability and proliferation of the cancer cell lines under analysis. We identified some possible mechanisms sustaining the antitumor activity of TE.

The leaves of *T. spicata* being rich in phenolic compounds received attention as possible nutraceutics for prevention of metabolic disorders and oxidative stress-related diseases [Avci et al., 2006; Akkol et al., 2009; Khalil et al., 2019]. Polyphenols, indeed, are good candidates as antiproliferative agents as they have been reported to exert a pro-apoptotic activity in many experimental models [Lambert et al., 2005]. Here, we demonstrated that ethanolic extract from *T. spicata* plays significant *in vitro* antitumor activity in different cancer cell lines, likely depending on the pool of its phenolic components with carvacrol being the most abundant one [Khalil et al., 2019]. Our findings showed that TE was more cytotoxic than pure carvacrol. TE ad dose of 100 μg/mL contains approximatively 25 μg/mL of CVL and reduced cell viability of more than 50%

in all tested cancer cell lines; whereas 25 μg/mL of pure CVL played no cytotoxic effect on all cancer cell lines. The higher cytotoxicity of TE compared to CVL as a single agent might depend on the synergism with some other phenolic compounds being present in the extract.

The mechanisms of action of antitumor drugs may involve different pathways including cell-cycle block, apoptosis induction and nitric oxide release. To clarify the mechanisms of TE activity we focused on MCF-7 (a breast cancer cell line). We found that TE exerted long term antitumor effects on MCF-7 cells through inhibition of colony formation and migration, and through reduction of the wound repair and cell proliferation recovery after treatment removal. Interestingly, the effects of TE were higher than those of pure carvacrol which was able to reduce colony formation with a lower efficacy than TE (-39% vs -66%, respectively), and did not play effect on cell proliferation recovery after treatment removal. The present results indicate that the ethanolic extract from *T. spicata*, at non-cytotoxic concentrations, might impair tumorgenicity of breast cancer cells *in vitro*.

Both TE and carvacrol exerted effects on ROS generation and NO release. Pure carvacrol increased the ROS levels and promoted apoptotic pathways resulting in a large early-apoptotic population (about 59%) that was detected by FACS analysis and micronucleus test. On the other hand, TE increase markedly not only the ROS levels, but also the NO release; these combined actions led to a stronger pro-apoptotic activity of TE compared to carvacrol resulting in the appearance of a late-apoptotic population (about 84%) as detected by FACS. The stronger pro-apoptotic activity of TE compared to carvacrol was confirmed by Bcl-2 expression. Of note, the role of NO in sustaining the apoptosis induction of TE was in accordance with previous data showing that the antitumor action of NO is dose-dependent: at low concentrations NO stimulated cancer cell growth, inhibited apoptosis and promoted cancer, whereas at higher concentrations NO inhibited cell proliferation and promoted apoptosis [Bal-Price, Gartlon, Brown, 2006; Villalobo, 2006]. NO may promote apoptosis through different mechanisms including nitrosylation of caspase 3 and cytochrome c [Schonhoff, Gaston & Mannick, 2003], inhibition of mitochondrial respiration [Moncada and Erusalimsky, 2002], and activation of death receptors [Razavi, Hamilton & Feng, 2005].

Many evidences suggest a possible association between inflammation and cancer progression [Mantovani, 2005]. NFκB and STAT3 are inflammation-linked transcription factors which modulate the ability of malignant cells to elude tumor-surveillance, and promote tumor

angiogenesis [Fan, Mao & Yang, 2013]. Our findings show that TE inhibited the NFκB signaling in MCF-7 cells through downregulation of phospho-p65, and this action may be a mechanism for suppressing metastasis of breast cancer cells. Moreover, we found that TE inhibited STAT3 phosphorylation, and this effect might be related to the pro-apoptotic effect of TE. In fact, STAT3 plays a crucial role in cancer cell proliferation, invasion and metastasis. STAT3 phosphorylation upregulates different anti-apoptotic genes such as those encoding Bcl-2, Bcl-XL [Rahaman et al., 2002; Konnikova, Kotecki, Kruger & Cochran, 2003]. Thus, STAT3 is a valuable target for cancer therapy [Zushi, Shinomura, Kiyohara & Matsuzawa, 1998; Lee et al, 2004].

CONCLUSION

In summary, this study demonstrates the potential antitumor activity of a panel of phenolic compounds contained in the edible plant *T. spicata* which is a typical herb of the Mediterranean wild flora [Al Hafi et al. 2017]. This finding might explain the use of *T. spicata* in Lebanon under form of the popular mixture “zaatar”. We propose that the ethanolic extract from *T. spicata* leaves might be a valuable source of novel natural antitumor compounds that might act by triggering cancer cell death and could be an effective supplement to traditional chemotherapies, although further investigations are needed.

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FIGURE LEGENDS

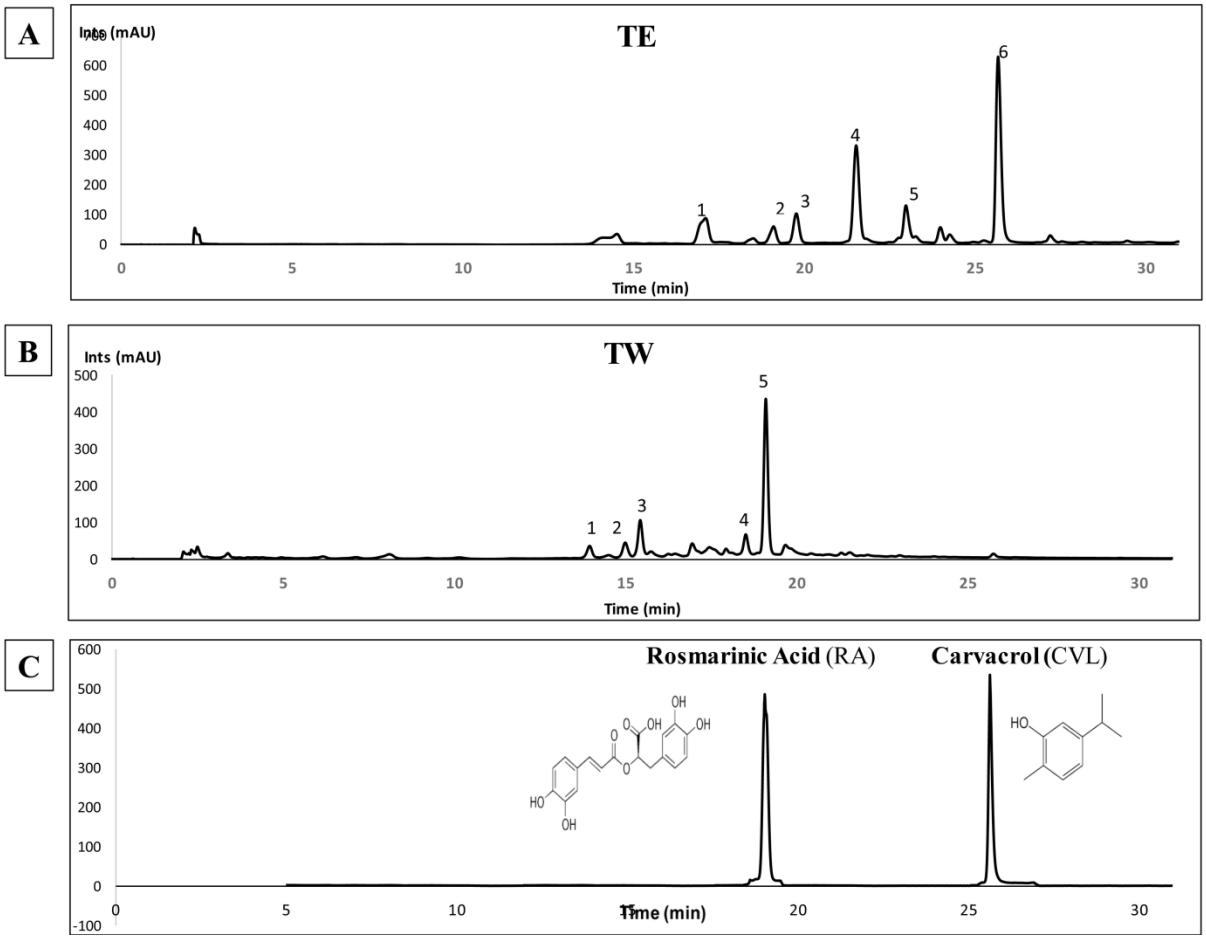

Figure 1: HPLC-UV chromatographic profiles were recorded at 280 nm for both extracts of *Thymbra spicata* and pure polyphenols; **(A)** Chromatogram of the ethanolic extract (TE) shows the following peaks: **1**: Eriodictyol derivate; **2**: Rosmarinc acid; **3**: Eriodictyol; **4**: Thymusin; **5**: Carvacrol. **(B)** Chromatogram of the aqueous extract (TW) shows the following peaks: **1**: Vicenin 2; **2**: Luteolin-O-diglucuronide; **3**: Salvalonic acid I; **4**: Rutin; **5**: Rosmarinc acid. **(C)** Chromatogram of the authentic standard compounds rosmarinic acid (RA) and Carvacrol (CVL), which are the most abundant components of TW and TE, respectively. For the HPLC-UV conditions in Materials and Methods section.

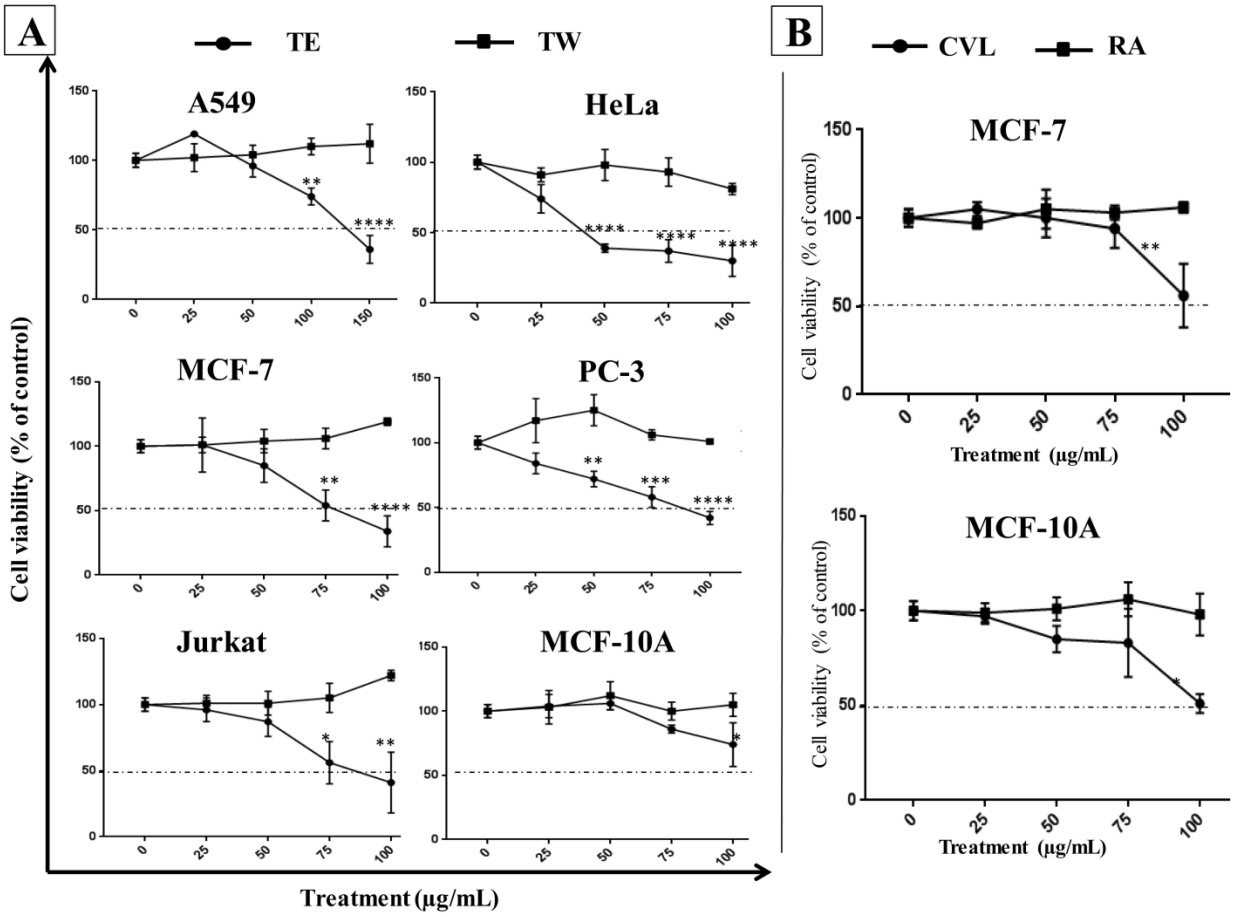

Figure 2: Effects of *Thymbra spicata* extracts on cell viability

(A) Exponentially growing A549, HeLa, Jurkat, MCF-7, PC-3 and MCF-10A cells were incubated in the absence (Ctrl) or in the presence of increasing concentrations (0-150 µg/mL) of ethanolic (TE) and aqueous (TW) extracts of *T. spicata* leaves for 24h. **(B)** Exponentially growing MCF-7 and MCF-10A breast cells were incubated for 24h in the absence (Ctrl) or in the presence of increasing concentrations ((0-100 µg/mL) of either Carvacrol (CVL) or Rosmarinic acid (RA), the most abundant components in TE and TW, respectively. Cell viability was detected by MTT assay as described in Materials and Methods. Data represent the mean of at least five independent experiments. Statistical analysis for cell viability data was performed using one-way ANOVA followed by Tukey's post-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****p<0.0001).

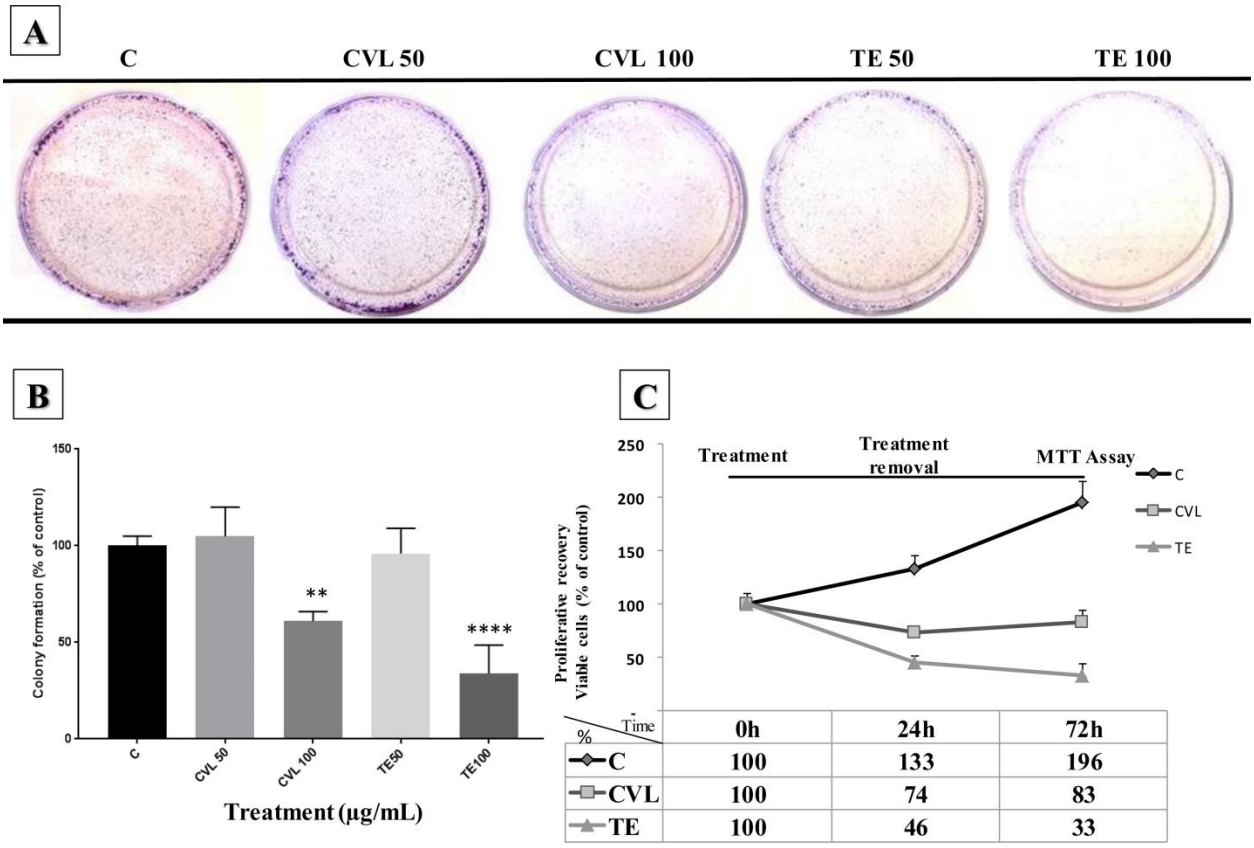

Figure 3: Effects of TE and Carvacrol on MCF-7 colony growth

MCF-7 cells were treated with CVL or TE (50 and 100 µg/mL) for 24h, and then allowed to grow for seven more days as described in Materials and Methods. Crystal violet staining allowed to detect the visible colonies (**A**) which were photographed by a Digital single-lens reflex Nikon D3300 2014. (**B**) Inhibition of colony growth was assessed by measuring the number of the colonies obtained in control and CVL- and TE-treated cells. Values are expressed as % of control. Data represent the mean of three independent experiments carried out in triplicate. **(C**) Inhibition of cell viability recovery after CVL and TE removal. MCF-7 cells were exposed to CVL or TE 100 µg/mL for 24 h, then, cells were washed to remove treatment and allowed to grow for another 48 h in fresh complete media. Cell viability was monitored using the MTT assay. Values are expressed as % of control. Data represent the mean of five independent experiments. Statistical analysis for cell viability data was performed using one-way ANOVA followed by Tukey's post-test (****$p < 0.001$, **$p < 0.01$).

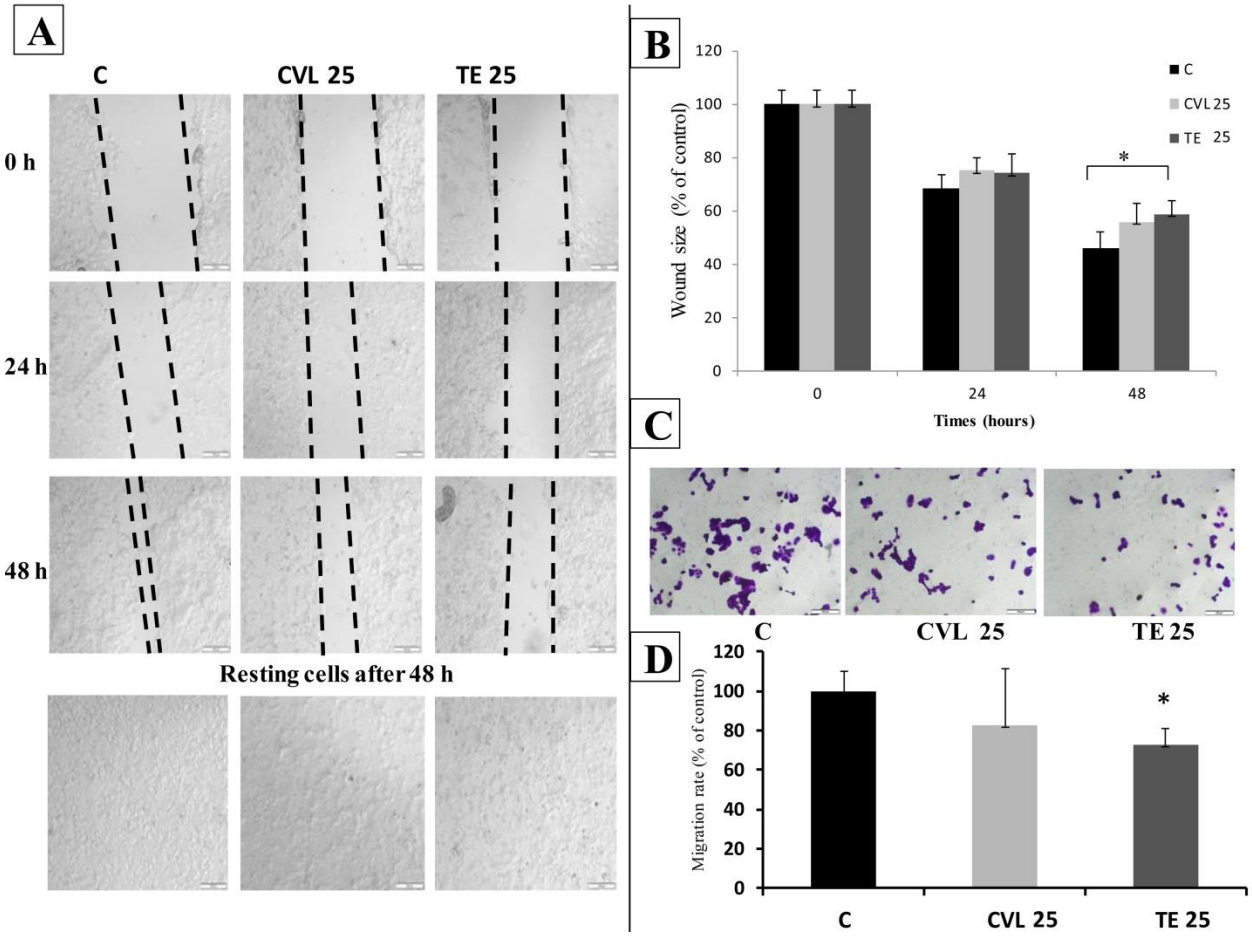

Figure 4: Effects of TE and Carvacrol on the migration of MCF-7 cells

(**A**) Representative images of confluent culture of MCF-7 cells wounded by scratching with a pipette tip, and then incubated in the absence (C) or in the presence of CVL and TE (25 µg/mL). The wound size was measured with a Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Magnification 10x; Bar: 100µm). (**B**) The graph reports the wound size after 24 and 48 h of growth. Values are expressed as % of control (**C**) Insert trans-well assay determined the migration of MCF-7 cells. MCF-7 cells were stained with crystal violet and photographed with an inverted microscope at x20 magnification. (**D**) The histogram indicates the number of migrating MCF-7 cells quantified by counting at least 5 random fields. Values are expressed as % of control. Data represent the mean of three independent experiments. Statistical analysis for cell viability data was performed using one-way ANOVA followed by Tukey's post-test (*$p < 0.01$).

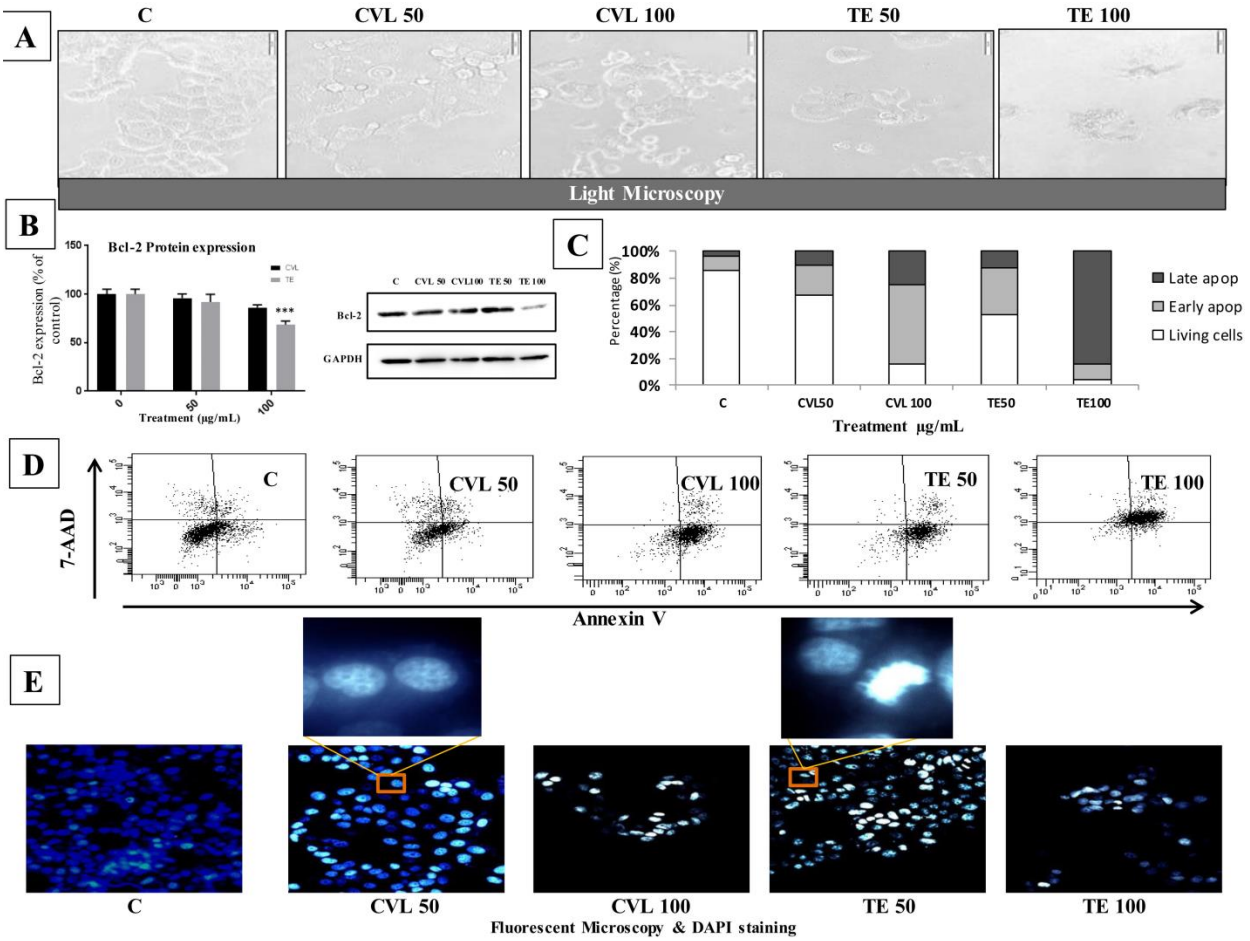

Figure 5: Pro-apoptotic effects of TE and Carvacrol

(A) Representative images of MCF-7 cells treated for 24h with increasing concentrations (50 and 100 µg/mL) of CVL or TE as described above. The changes in cell morphology were observed under a Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Magnification 10x; Bar: 100µm). **(B)** Western Bot analysis assessed the Bcl-2 protein expression in MCF-7 cells incubated in the absence (Ctrl) or in the presence of CVL and TE (50 and 100 µg/mL). Data represent the mean of at least three independent experiments. Statistical analysis for cell viability data was performed using one-way ANOVA followed by Tukey's post-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **(C-D)** The apoptotic fraction in each cell sample was quantified by Annexin V/7-AAD FACS analysis as described in Materials and Methods. Cells were incubated in the absence (Ctrl) or in the presence of CVL and TE for 24 h. **(E)** The presence of micronuclei was assessed by Fluorescence Microscopy of DAPI-stained cells under an inverted Olympus IX53 microscope equipped with a CCD UC30 camera and (Magnification 20x; Bar: 50µm).

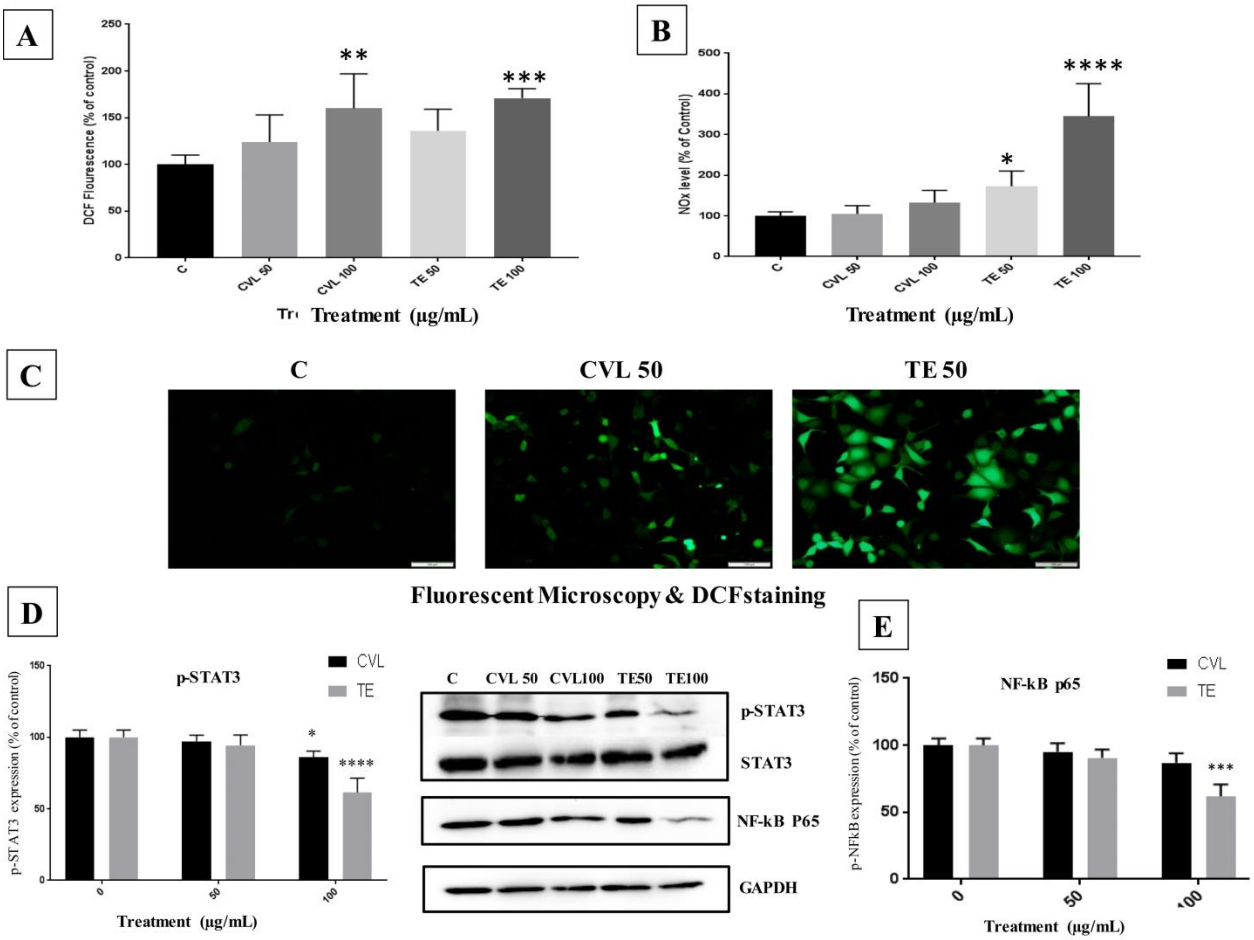

Figure 6: Effects of TE and Carvacrol on: ROS and NO production; STAT3 and NFκB activation

(A) Effect of TE and CVL on the intracellular levels of ROS was measured with H2-DCFDA staining as described in Materials and Methods. Cells were incubated in the absence (Ctrl) or in the presence of CVL and TE (50 and 100 µg/mL) and the DCF fluorescence was quantified by spectrofluorimeter assay of DCF-stained cells. Values are expressed as % of control. **(B)** Nitric oxide (NO) production was quantified in the medium of CVL and TE-treated (50 and 100µg/mL) and untreated MCF-7 cells as µmol $NaNO_2$/mg sample protein by Griess reaction. **(C)** The intracellular ROS production was visualized *in situ* by fluorescence microscopy of DCF-stained cells. Images were acquired at Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Magnification 20x; Bar: 50µm). (**D**) The level of phospho-STAT3 and of phosphor-NFκB (**E**) were assessed in MCF-7 cells incubated in the absence (Ctrl) or in the presence of CVL and TE (50 and 100 µg/mL) for 24 h. GAPDH was assessed as loading control.

Data represent the mean of three independent experiments. Values are expressed as % of control**.** Data represent the mean of five independent experiments. Statistical analysis for cell viability data was performed using one-way ANOVA followed by Tukey's post-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.001$).

- **CHAPTER 3.**

Repressive effect of Lebanese *Rhus coriaria* fruit extracts on microglial cells-mediated inflammatory and oxidative stress responses

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ABSTRACT

Background: *Rhus coriaria*, a herbal shrub found in the Middle East region, is well known for its biological activities and pharmacological potential. Nevertheless, the effect of *R. coriaria* on microgial cells, immunocompetent phagocytes which play an important role in neuronal protection or damage, is still poorly characterized.

Objectives: In the present study, phytochemical analysis as well as antioxidant and anti-inflammatory potential assessment of *R. coriaria* extracts on BV-2 microglial cells was performed.

Material and Methods: *R. coriaria* fruits were dissolved in two different solvents: distilled water and ethanol. Phytochemical screening was performed to determine the bioactive components. Anti-oxidant potentail was assessed by DPPH method (2,2-diphenyl-1-picrylhydrazyl) and DCF assay (2',7'-dichlorofluorescein). TNFα and IL-10 mRNA levels were quantified by quantitative real-time PCR (qRT-PCR). NFκβ protein levels were determined by western blot.

Results: We showed that *R. coriaria* ethanolic and aqueous extracts contain high phenolic and flavonoid contents. Interestingly, these extracts exerted a potent anti-inflammatory potentail on LPS-insulted BV-2 cells manifested by: (1) suppressing TNFα mRNA levels ; reducing NFκβ activation ; and (3) enhancing IL-10 transcription levels. These extracts also showed prominent anti-oxidative stress capacity due to their ability to rescue cell viabilty and reduce reactive species (ROS/RNS) production in H_2O_2-insulted cells.

Conclusion: Our findings suggest that *R. coriaria* might carry therapeutic potentail against neurodegenerative diseases

Keywords: Microglai; *Rhus coriaria*; anti-oxidant; anti-inflammatory; neurodegenerative diseases.

INTRODUCTION:

Neuro-inflammation, underlying neuronal injury, is one of the major hallmarks of many neurodegenerative diseases including Alzheimer disease, Parkinson disease, multiple sclerosis. Under pathological conditions, the progression of the neurodegenerative disease is accompanied with mitochondrial failure and dysfunction, which in turn leads to Reactive oxygen/nitrogen species (ROS/RNS) generation, thereby causing oxidative stress (Kim et al., 2015; Gelders et al., 2018). ROS-mediated oxidative injury contributes to DNA damage and cell death, thus resulting in hyperactivation of microglial cell-mediated inflammatory cascades by enhancing the expression of pro-inflammatory cytokines [Tumor Necrosis Factor alpha (TNF-α), Interleukin 1 beta (IL-1β) and IL-6) and mediators [Inflammation-inducible isoform of nitric oxide synthase (iNOS) and Cyclooxygenase-2 (COX-2)] (Hansen et al., 2018). Microglia, the principal cells involved in the innate immune response that defend against central nervous system (CNS) insults, play essential roles in the maintenance of homeostasis and responses to neuroinflammation conditions (Wolf et al., 2017). Intriguingly, microglial cells do not acquire unique morphology; instead, they alter their phenotype and function according to the surrounding environmental signals (Tam & Ma. 2014). For instance, in response to lipopolysaccharide (LPS) stimulus, microglia adopt M1 classical phenotype and exert a pro-inflammatory role via secreting TNF-α, IL-1β, IL-6 and ROS/RNS (Liu et al., 2019). Thus, M1 microglia contribute to aggravation of oxidative stress and inflammation. On the other hand, microglia can be alternatively activated to acquire an anti-inflammatory M2 phenotype, thus secrete anti-inflammatory cytokines such as IL-10 and transforming growth factor beta (TGF-β) (Stansley et al. 2012; R. Orihuela et al. 2015). Tailoring the phenotype and function of microglia cells via controlling the surrounding environmental conditions is of great interest to enhance their therapeutic potential against inflammation-based neuropathologies.

Plants have been an important source for pharmacologically active compounds, especially polyphenols which represent the most important bioactive compounds of the medical plants and have been investigated as candidates to prevent/treat various diseases [García-Conesa, 2017]. *Rhus coriaria* L. (Anacardiaceous), known as Sumac, is one of the herbal shrubs that are found in the Middle East countries including Lebanon. Besides its nutritional value, Sumac has been known for its remarkable medicinal potential where it is used, in the Middle East region, for the treatment of stroke, hypertension, haematemesis, ophthalmia, stomach ache, diabetes, atherosclerosis, and liver diseases (Khalilpour et al., 2017, 2019). The phytochemical compounds of sumac have been characterized using HPLC–DAD–ESI-MS/MS method, Abu-reidah et al., (Abu-reidah et al., 2015) identified 211 phenolic nature compounds and other phytochemicals including organic acids, phenolic compounds conjugated with malic acid derivatives, terpenoids and other compounds. Many studies suggest that *R. coriaria* extracts and phytochemicals exert a wide range of biological and pharmacological activities including anti-tumor (Athamneh et al., 2016; El Hasasna et al., 2016), anti-diabetic (Mohammadi et al., 2010), antihypertensive (Anwar et al., 2016), anti-oxidative (Candan and Sokmen, 2004) and anti-inflammatory (Khalilpour et al., 2019; Momeni et al., 2019). Despite of this, the effect of *R. coriaria* on the nervous system physiology is still poorly characterized. Accordingly, we examined, in this study, the ability of *R. coriaria* fruits extracts to suppress the pro-inflammatory and oxidative stress responses mediated by the murine BV2 microglial cells. The outcome of this study highlights a promising therapeutic potential of *R. coriaria* for treating neurodegenerative diseases.

MATERIALS AND METHODS:

2.1. Plant collection and extraction:

Fruits of *Rhus coriaria* were collected from a private farm located in Maifadoun, Nabatieh Governorate, Lebanon (470 m above sea level) at 33°21’19.9”N 35°28’22.4”E. *R. coriaria* fruits were dried in shade, at room temperature, for three weeks. The grinded materials were then subjected to solvent extraction with 100% ethanol or double-distilled water (ddH_2O), using standard procedures. Briefly, 20 grams of dried material were dissolved in 100 ml of each

solvent and sonicated for one hour at 60°C. Solvents were then evaporated in a Rotavac vario power unit (Heidolph Instruments, Schwabach, Germany), and then freeze-dried in Alpha 1-4 LD plus lyophilizer (CHRIST, Osterode am Harz, Germany). The extraction yield was then calculated and the dry fractions were stored at -20°C until use.

2.2. Qualitative phytochemical analysis

R. coriaria fruits extracts were screened for the presence of secondary metabolites such as flavonoids, alkaloids, saponins, steroids, cardiac glycosides, phenolics, and tannins as described by previous method (Kumari et al., 2018).

2.3. Quantification of total phenolic compounds (TPC):

The *Folin Ciocalteau* method was used to estimate total phenolic content (TPC), according to Singleton and Rossi, (Singleton and Rossi, 1965) with slight modifications. Briefly, 100 µl of ethanolic and aqueous extract (1 mg / mL) were prepared. Gallic acid (GA) was used as standard to determine concentrations of phenols. Different concentrations (0, 5, 10, 20, 35, 50 µg / mL) of GA were prepared and mixed with 0.5 ml of *Folin Ciocalteau* reagent (Dilution 1/10). After 5 minutes, the sodium bicarbonate (Na_2CO_3) was added. The mixture was incubated in the dark for 30 minutes at room temperature. Results of obtained blue solution were measured against blank by U-2900 760 UV-Vis Spectrophotometer 200V (Hitachi High-Technologies, Japan). Values were expressed in mg gallic acid equivalent (GAE) per g of dry weight of plant extract powders.

2.4. Quantification of total flavonoid compounds (TFC):

The Aluminum Trichloride method was used to estimate the total flavonoid content (TFC) as previously described by Quettier-deleu (Quettier-deleu et al. 2000) with minor changes. Briefly, 1 mL of each extract (from stock of 5 mg / mL to obtain a final concentration of 0.5 mg / mL) and 1mL of each of five concentrations (1, 2, 5, 10, 15 µg / mL) of quercetin, used as a standard to determine the concentration of flavonoids, were prepared and mixed with 1 ml of methanolic solution of aluminum chloride (2%). Following incubation (1 hour at room temperature in the dark), the absorbance of all samples was determined at 415 nm using a U-2900 UV-Vis spectrophotometer 200 V (Hitachi High Technologies, Japan). The results were expressed in mg

of quercetin per g dry weight powders. It is important to note that 1 mL of solvent (ethanol and water) mixed with 1 mL of 2% of the solution methanolic aluminum chloride were used as blank before reading the samples.

2.5. 2,2-diphenyl-1-picrylhydrazyl (DPPH radical scavenging assay

The antioxidant potential was determined by the DPPH radical scavenging activity. Increasing concentrations of extracts (0, 10, 20, 50, 80, 100 µg / mL) were prepared. 1 ml of each of the prepared dilutions was added to 1 ml of DPPH reagent (0.0026g in 50 mL methanol). This mixture was then incubated in the dark for 30 min at room temperature. The absorbance was then measured at 517 nm by U-2900 UV-Vis spectrophotometer 200 V (Hitachi High-Technologies, Japan). Ethanol and distilled water were used as blanks for the ethanol and water extracts, respectively. Ascorbic acid was used as a positive control. Finally, the DPPH trapping capacity of plant extracts was calculated using the following equation: % Scavenging activity = [(Abs control -Abs sample)] / (Abs control)] x 100 (Khalil et al., 2019).

2.6. Cell culture and treatment:

BV-2 cells, a Murine microglial cells, were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium High Glucose (D-MEM) supplemented with L-Glutamine, 10% FBS and penicillin (100 U / mL) and streptomycin (100 mcg /mL). For treatments, cells were grown until 80% confluence. To induce oxidative stress and inflammation conditions, BV-2 cells were incubated for 24 h with H_2O_2 at 50 µM, or LPS (Sigma, Beirut, Lebanon) at 1µg/mL, Escherichia coli K-235 > 55.104 U/mg, respectively. Thereafter, cells were incubated for 24h in fresh medium containing the extracts at different concentrations (0, 25 and 50 µg/mL). For each experiment, treatment was performed in quadruplicates.

2.7. Cell viability assay:

BV-2 cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method according to Hussain et al, 1993. Stock solutions (50 mg/mL) were prepared in dimethyl sulfoxide (DMSO) for the ethanol extract (RE) and sterile double distilled water ddH_2O for the water-soluble extract (RW). For analysis, cells were seeded in 96-well plate (10^4 cells per well), in complete medium. After 24 h (confluency ≈ 75%), BV-2 cells were

incubated for another 24 h with H_2O_2 (30 µM). Thereafter, cells were incubated for 24h with extracts at different concentrations (µg/mL). At the indicated times, 20 µL of MTT reagent (5.0 mg/mL) was added and incubated at 37°C for 4 h. The unreacted MTT dye was then removed by aspiration and 100 µL of acidified isopropanol was added to solubilize purple formazan crystals within metabolically active cells. The absorbance of sample was recorded at 570 nm by multi detection microplate reader (Biotek Instrument, Winooski, VT, USA). Data are expressed as percentage of cell viability with respect to control. For each experiment, treatment was performed in quadruplicates.

The inhibitory rate was calculated by the following formula:

$$\text{Inhibitory rate (\%)} = (1- At/Ac) \times 100$$

Where *At* and *Ac* were the absorbance values of the treatment and control wells, respectively.

2.8. ROS production

The oxidation of the cell-permeant 2'-7' dichlorofluorescin diacetate DCF-DA (Sigma, Beirut, Lebanon) to 2'-7'dichlorofluorescein (DCF) allowed to quantify *in situ* the production of H_2O_2 and other ROS. Stock solution of DCF-DA (10 mM in DMSO) was prepared and stored at -20°C in the dark. At the end of treatment, the cells were washed with PBS and incubated with 10 µM of DCF-DA at 37°C. After 30 min, cells were washed once with PBS, and the fluorescence intensity of the oxidized form, 2′,7′-dichlorofluorescin (DCF), was measured in a microplate reader at 492 nm (excitation) and 520 nm (emission). Fluorescence values were calculated after background subtraction (using identical conditions without DCF).

2.9. Nitrite/Nitrate (NOx) Levels

NO production was measured by spectrophotometric measurement of the end products, nitrites and nitrates, using the Griess reaction [Green et al. 1982]. After treatments, nitrite accumulation (µmol $NaNO_2$/mg sample protein) was calculated against a standard curve of sodium nitrite

($NaNO_2$). All spectrophotometric analyses were carried out at 25°C recording absorbance at 546 nm.

2.10 Protein extraction and Western blot analysis:

BV-2 cells (1×10^6) were seeded in 10 cm petri dishes and grown for 24 h. At the end of treatments, cells were washed, scraped, pelleted and lysed in RIPA Buffer supplemented with protease inhibitor cocktail (Sigma Aldrich, Beirut, Lebanon) and phosphatase inhibitor cocktail. After incubation for 15 min on ice, cell lysates were centrifuged at 14,000 rpm for 20 min at 4°C. Protein concentration of lysates was determined by Lowry protein assay kit (BioRad, Beirut, Lebanon). Aliquots of 30-40 μg of total cell lysate were resolved into 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Biorad, Beirut, Lebanon) and blocked for 1 h at room temperature with 5% non-fat dry milk in PBST (phosphate buffered saline with 0.1% Tween 20), anti-phos-RelANFkB p65, and anti-hGAPDH antibodies were purchased from R&D systems (Minneapolis, USA). Incubation with specific primary antibodies was performed in blocking buffer for overnight at 4 °C. Horseradish peroxidase-conjugated anti-IgG was used as secondary antibody for 1 hour at room temperature Immune complexes were visualized using an enhanced chemiluminescence Western blotting analysis system (Bio-Rad ChemiDoc XRS System) and then the band optical densities were quantified against the GAPDH band using ImageJ free software (http://imagej.nih.gov/ij/).

2.11 mRNA quantification by quantitative Real time-PCR (qRT-PCR):

Twenty-four hours after treatment with plant extracts, total RNA was extracted from cell cultures using Trizol reagent (Sigma, Beirut, Lebanon). RNA (1 μg) was reversely transcribed to cDNA. The cDNA was amplified by PCR, using the SYBR green master mix (Sigma, Beirut, Lebanon) and two specific oligonucleotide murine primers (Macrogen,Korea). The primer sequences listed in table 1. Using LineGene software, each PCR cycle was run for 15s at 95°C and 1min at 60°C. mRNA levels were normalized to GAPDH. Gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method.

2.12 Statistical analysis:

Data are means ± S.D. of at least three independent experiments. Statistical analysis was performed using ANOVA with Tukey's post-test (GraphPad 7.0 Software, Inc., San Diego, CA, USA).

RESULTS:

3.1. Phytochemical screening of *R. coriaria L.* fruit extracts .

Phytochemical screening of *R. coriaria* fruits extracts unravelled important bioactive components which are listed in Table 2. Terpenoids, Quinones, Sterol and Steroids, Diterpenes, Phenols, Proteins and Amino acids, Flavonoïdes, Resines, Anthocyanins, Flavanones and Cardiac glycosides were identified in both, ethanol and aqueous extracts (Table 2). Alkaloids as well as Oil and fatty acids were found in ethanol extracts (Table 2). Reducing sugars, Anthraquinones, Tannins and Saponins were identified only in aqueous extract (Table 2).

Total phenolic and flavonoid contents (TPC and TFC) in *R. coriaria* ethanolic (RE) and *R. coriaria* aqueous extracts (RW) are shown in Figure 1. TPC in ethanol extract (RE) was significantly higher (139 mg GAE / g) than in aqueous (RW; 48.5 mg GAE / g) extract. This result is expected since phenol is more soluble in organic solvents (such as ethanol and methanol) than the other solvents (such as water or hexane). On the other hand, TFC was higher in aqueous (7.48 mg EQ / g) than ethanol (5.15 mg QE / g) extract.

3.2. Radical scavenging activity of *R. coriaria* extracts.

DPPH free radical scavenging assay was performed to assess the cell-free antioxidant activities of ethanolic and aqueous extracts of *R. coriaria* fruits (Table 3). It is noteworthy that ascorbic acid was used as a positive control. Our results showed that the scavenging effects on DPPH radical were in the following order: ascorbic acid (IC_{50}=6.5 µg /mL) > ethanolic extract (IC_{50}=20 ± 2.6 µg/ml) > aqueous extract (IC_{50}=54±2.77 µg/ml) (Table 3).

3.3. Cytotoxic effect of *R. coriaria* extracts on murine microglial BV-2 cells

In order to assess the possible cytotoxic effect of *R. coriaria* ethanolic and aqueous extracts on murine microglial BV-2 cells, MTT assay over a range of concentrations (0.1 to 500 µg/mL) was

performed (data not shown). Cell viability was comparable in extract-treated versus control cells in the different used concentrations, indicating absence of cytotoxic effect against murine microglial BV-2 cells. Figure 2 shows results of two selected concentrations that we used for further experiments (25µg/mL and 50µg/mL) of ethanolic (RE) and aqueous (RW) extracts. After 24 hours, cell viability was not affected in neither RE- nor RW-treated cells (Figure 2A). After 48 hours, cell viability of RW treated-cells was not influenced; whereas a slight non-significant decrease in BV-2 cells viability was observed following treatment with either 25 or 50µg/mL of ethanolic extract RE (Figure 2B).

3.4. Anti-oxidative stress effect of *R. coriaria* extracts on oxidative injured microglial cells

A very typical approach to induce oxidative stress in microglial cell is treatment with hydrogen peroxide. The effect of ethanolic and aqueous extracts on H_2O_2-treated cells' viability was evaluated using MTT assay. Control cells (not exposed to H_2O_2) viability increased gradually over the time period (days 1, 2 and 3) (Figure 3). On the other hand, H_2O_2-treated cells showed a significantly lower viability rates at days 2 and 3 (Figure 3). Interestingly, supplementing H_2O_2-treated cells with ethanolic or aqueous extracts (50 µg/mL) promoted the recovery of viability (at day 3) where it increased significantly by 32% when treated with ethanolic extract than H_2O_2-treated cells (#$p<0.05$) and 16% when treated with aqueous extract than H_2O_2-insulted cells ($p<0.01$) (Figure 3).

The anti-oxidative potentail of *R.coriaria* extracts was further assessed by Dichlorofluorescein (DCF) assay in which ROS production was measured. Figure 4 showed that ROS production in H_2O_2-treated BV-2 cells was significantly higher as compared to control cells (%*$p<0.05$). Interestingly, treatment with ethanol (50µg/mL) or aqueous extract (50µg/mL) significantly decreased ROS production (####$p<0.0001$) in H_2O_2-treated BV-2 cells.

3.6-Anti-inflammatory effect of *R. coriaria* extracts

To assess the anti-inflammatory capacity of *R. coriaria* extracts, BV-2 cells were treated, for 24 hours, with either LPS (100ng/ml) alone (control), or LPS together with 50 µg/ml of ethanol or aqueous extracts where TNFα (pro-inflammatory cytokine) and IL-10 (anti-inflammatory cytokine) mRNA levels were quantified by quantitative Real-Time PCR (qRT-PCR) (Figure 5). In the case of TNFα, the high mRNA levels exhibited by LPS-treated cells were strikingly

reduced upon treatment with either ethanolic or aqueous extracts (Figure 5A). On the other hand, the low IL-10 transcript levels observed in LPS-treated cells were significantly induced upon treatment with either ethanol or aqueous extracts (Figure 5B).

The NO production, a classical marker for inflammation, was increased markedly in BV-2 cells injured with hydrogen peroxide (+147%; $p \leq 0.0001$) with respect of control (Fig6A). At both concentrations (25 μg/mL and 50 μg/mL) the ethanolic extract RE was able to counteract the stimulation of NO release compared to H_2O_2-insulted cells ($p \leq 0.0001$), no significant reduction of NO was shown upon RW treatment. (Figure 6A).

NFκβ (P65) expression levels are usually used as readout of inflammatory response. The anti-inflammatory effect of *R. coriaria* extracts was further evaluated by examining NFκβ activation levels by Western Blot analysis in the same experimental conditions (Figure 6B). Our results showed that the induced NFκβ phosphorylation levels exhibited by LPS-treated cells were significantly lowered upon treating cells with ethanolic extract. On the other hand, only a minor non-significant decrease was observed in cells exposed to aqueous extract (Figure 6B).

DISCUSSION

Rhus coriaria, a wild edible plant in the Mediterranean region, is well known for its biological properties (including anti-oxidant, anti-bacterial, and anti-tumor activities) and pharmacological potential (treatment of distinct health disorders including hypertension, diabetes and atherosclerosis). Currently, only few information, if any, are available about the influence of *R. coriaria* extracts on CNS biology. The major outcome of this study highlighted a promising therapeutic value of *R. coriaria* fruits, against inflammation-based nervous-system related pathologies, due to its ability to repress the pro-inflammatory and oxidative stress activities mediated by the murine BV2 microglial cells.

In this study, our preliminary phytochemical analysis identified the presence of different bioactive components with therapeutic value. For instance, both the ethanolic and aqueous extracts of *R. coriaria* fruits showed a high content of phenols and flavonoids which may responsible for their anti-oxidation, anti-inflammation and free radical scavenging properties.

Indeed, previous reports showed that phenol/flavonoid rich extract of *R. coriaria* exhibited a potential anti-inflammatory, anti-diabetic and hepatoprotective activites (Mohammadi et al, 2010; Asgarpanah & Saati, 2014; Salimi et al. 2015).

Currently, the effect of *R. coriaria* extracts on behavior of nervous system components, in particular the microglial cells (BV-2 cells), is still poorly characterized. A recent report showed that ethanol extract of *R. coriaria* has an antioxidant, anti-inflammatory, and neuro-protective effect *in vivo* where it inhibited the inflammatory response to ischemia in an optic neuropathic model (Khalilpour S et al. 2018). However, no experiments provided the effect of this plant on microglial cells (BV-2). Hence, we examined in our study the anti-inflammatory and anti-oxidant effect of *R. coriaria* fruits extracts on BV-2 cells.

We showed that LPS of high concentration (1µg/ml) induces an increase in BV-2 cells-mediated expression of TNFα (pro-inflammatory cytokine) and decrease in IL-10 (anti-inflammatory cytokine) levels. This is consistent with a previous report showing that 4 and 8 hrs exposure of BV-2 cells to LPS (1µg/ml) leads to a significant elevation in TNFα mRNA level and decrease in IL-10 levels (Awada et al. 2014). LPS stimulates the expression of neurotoxic cytokines such as TNFα and IL-6 through an inflammatory cascade involving Nuclear Factor Kappa Beta (NFκβ) transcription factor. In our study, we showed that NFκβ p65 is highly expressed in cells treated with LPS. This is consistent with a previous report demonstrating, using confocal immunofluorescence, that the nuclear abundance of NFκβ p65 increases significantly following LPS stimulation (Badshah et al., 2016). The increased expression of TNFα is explained by the fact that microglial cells have Toll-like receptors 4 (TLR4) which recognize LPS. Upon LPS binding, TLR4 is activated, leading to the degradation of inhibitor κβ (Iκβ) proteins and eventual translocation of NFκβ to the nucleus. NFκβ can then drive the expression of pro-inflammatory cytokines including TNFα (Badshah et al., 2016). In our study, we observed a significant anti-inflammatory capacity of ethanolic and aqueous extracts of *R. coriaria* fruits on microglial cells (BV-2), due to their ability to repress NFκβ phosphorylation levels and TNFα mRNA transcripts, and to induce IL-10 transcription. This prominent anti-inflammatory capacity could be attributed to the high phenolic content in these extracts.

In this study, we observed that treatment of BV-2 cells with H_2O_2 induces cytotoxicity by decreasing cell viability and promotes oxidative stress by producing ROS as proved in MTT

assay and DCF fluorescence assay respectively. These observations are in accordance with a recent report in which exposing BV2-cells to H_2O_2 (75 µM) activates the cells by direct oxidation which is mediated by the production of superoxide (Cobourne-Duval et al. 2016). Although ROS are considered important for neuronal function and development, abnormal increase of ROS (e.g. oxidative stress) can provoke extensive protein oxidation and lipid peroxidation; thus, mediating oxidative damage and neuronal degeneration that retard synaptic signaling and brain plasticity (Salim et al., 2017). *R. coriaria* fruits extract led to a significant rescue of oxidative stress and cell dysfunction by decreasing ROS production and NO release (indicator of microglia inflammation and oxidative damage). The extracts were also able to recover cell viability after H_2O_2-insult and protected BV-2 cells from H_2O_2-induced cytotoxicity. The obtained high anti-oxidant property of *R. coriaria* fruits extracts against oxidative stress in BV-2 cells could be explained by the anti-radical scavenging capacity of both extracts. We would further investigate the pathways implicated in this anti-oxidant activity by studying the expression of genes coding for anti-oxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and Glutathione peroxidase (GPx). Moreover, the anti-oxidant potential of *R. coriaria* fruits extracts should be tested in vivo. Overall, our results suggest a promising therapeutic potential for *R. coriaria* against neurodegenerative diseases.

CONCLUSION:

In conclusion, the present study reveals that *R. coriaria* fruits contain important bioactive molecules and can significantly supress oxidative stress and inflammatory responses mediated by microglial cells. *R. coriaria* could therefore hold therapeutic potential for treating neuropathic diseases.

ACKNOWLEDGEMENTS AND FUNDING

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ABBREVIATIONS

Abs: Absorbance;

ALCL3: Aluminum Chloride;

CNS: Central Nervous System;

DCF: 2',7'- dichlorofluorescein;

DMEM: Dulbecco's Modified Eagles Medium;

DPPH: 2,2-diphenyl-1-picrylhydrazyl;

GA: Gallic Acid;

GAE: Gallic Acid Equivalent;

H_2O_2: Hydrogen Peroxide;

IC50: Half maximal inhibitory concentration;

IL10: Interleukin 10;

LPS: Lipopolysaccharide;

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;

NFκβ: Nuclear Factor Kappa Beta;

PBS: Phosphate Buffer Saline;

QE: Quercetin Equivalent;

qPCR: quantitative Polymerase Chain Reaction;

RE: *R. coriaria* ethanolic extract

ROS: Reactive Oxygen Species;

RNS: Reactive Nitrogen Species;

RW: *R. coriaria* aqueous extract

TFC: Total Flavonoid Content;

TLR4: Toll-like receptors 4 ;

TNFα: Tumor necrosis factor alpha;

TPC: Total Phenolic Content;

UV: Ultra-Violet

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TABLES:

Table 1: Primers sequences

Gene	Primers (F: Forward and R: Reverse)
Glyceraldehyde-3-phosphatedehydrogenase (GAPDH)	F: 5′-TTCACCACCATGGAGAAGGC-3′
	R: 5′-GGCATG- GACTGTGGTCATGA-3′
Tumor necrosis factor (TNFα)	F: 5'-TGGCCTCCCTCTCATCAGTT- 3'
	R: 5'-GCTTGTCACTCGAATTTTGAGAAG- 3'
Interleukine 10 (IL-10)	F: 5'-ACCTCCTCCACTGCCTTGCT- 3'
	R: 5'-GGTTGCCAAGCCTTATCGGA- 3'

Table 2: Results of phytochemical analysis. The table presents the bioactive components present (+) or absent (-) in *R. coriaria* extracts.

Bioactive Components	Ethanolin extract RE	Aqueous extract RW
Reducing sugar	-	+
Anthraquinones	-	+
Proteines and amino acids	+	+
Phlabotannins	-	-
Alkaloids	+	-
Tannins	-	+
Resines	+	+
Terpenoïds	+	+
Flavonoïdes	+	+
Quinones	+	+
Sterols et steroïds	+	+
Diterpenes	+	+
Anthocyanins	+	+
Flavonones	+	+
Lignins	-	-
Cardiac glycosides	+	+
Saponins	-	+
Phenols	+	+
Oil and fatty acids	+	-

Table 3: DPPH radical scavenging activities. The half maximal inhibitory concentration (IC50) at which antioxidant activity is 50% was calculated. The data are reported as mean ±SD

Samples	IC50 (µg/ml)
Ascorbic acid	**6.5 ± 1.5**
RE	**20 ± 2.6**
RW	**54±2.77**

FIGURE LEGENDS:

Figure 1

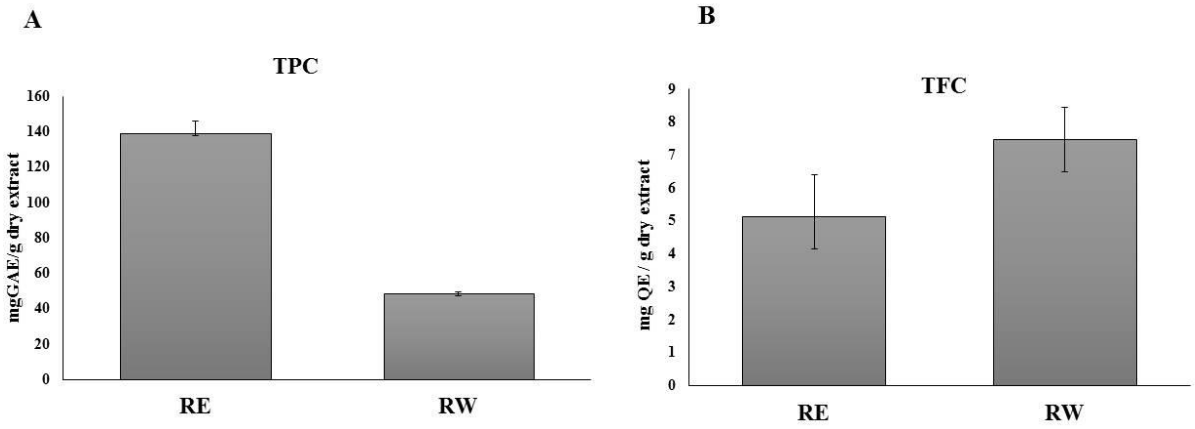

Figure 1. Total phenolic and flavonoid contents in *R. coriaria* ethanolic (RE) and aqueous (RW) extracts. (A) The total phenolic content (TPC) was expressed as mg of Gallic Acid Equivalent (GAE) per g of dry extract, **(B)** the total flavonoid content (TFC) was expressed as mg of Quercetin Equivalent (QE) per g of dry extract.

Figure 2

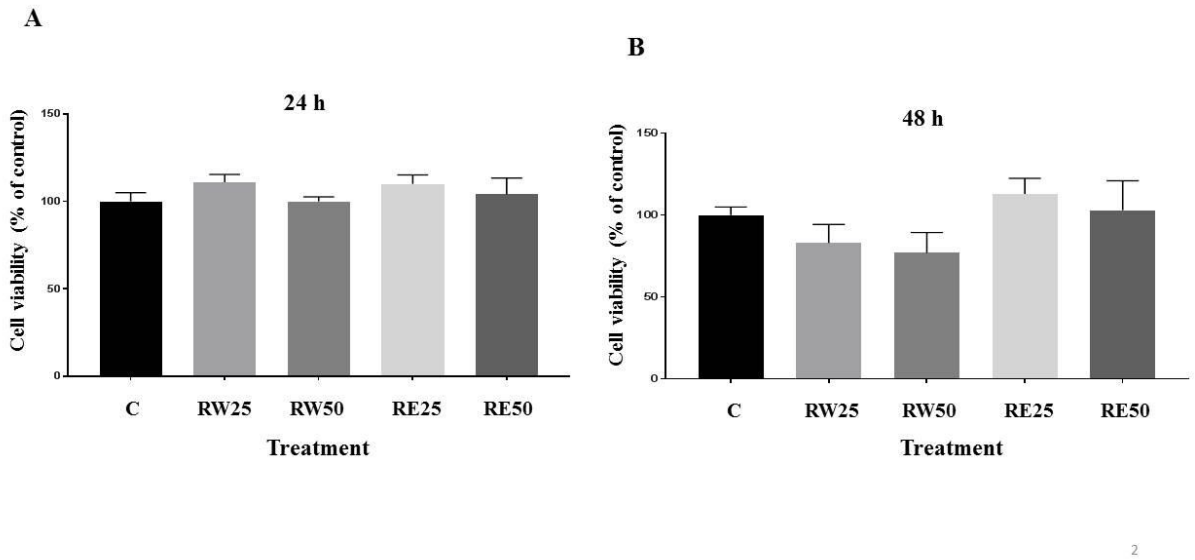

Figure 2. Effect of *R. coriaria* ethanolic (RE) and aqueous (RW) extracts on viability of microglial cells (BV-2). BV-2 cells were treated with either 25µg/ml or 50 µg/ml of either ethanolinc or aqueous extracts for 24 **(A)** or 48 hours **(B).** Cell viability was examined by MTT assay. The results were expressed as percentage of control (untreated cells). Data represent an average of three independent experiments and reported as mean ± SD.

Figure 3

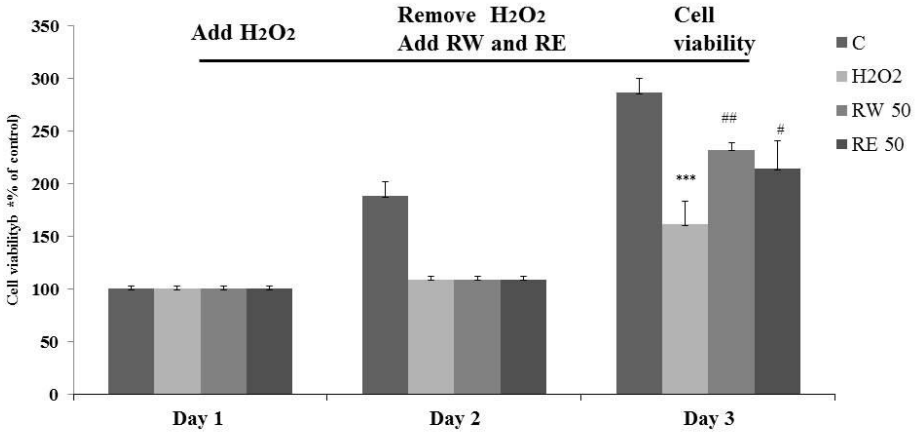

Figure 3. Cytoprotective Effect of *R. coriaria* extracts against H_2O_2-induceed cytotoxicity in microglial cells' (BV-2). Cells were treated with H_2O_2 for 24 hours followed by extracts treatment for 24 hours. Cell viability was assessed by MTT. Values are mean ± S.D from a least three independent experiments. Significant differences are denoted by symbols: C *vs* H_2O_2 insulted cells ***$p \leq 0.001$ and H_2O_2 insulted cells *vs* extracts, #$p \leq 0.05$, ##$p \leq 0.01$.

Figure 4

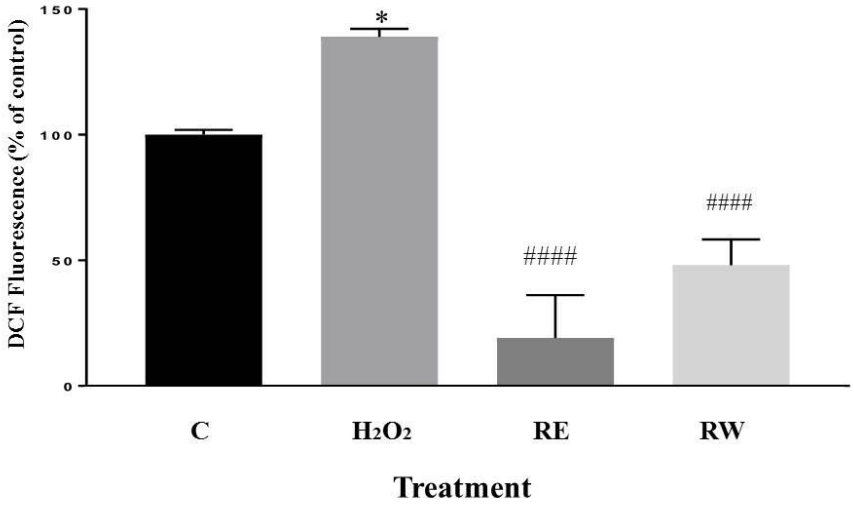

Figure 4. Effect of *R. coriaria* extracts on ROS production in H_2O_2-injured BV-2 cells. DCF BV-2 cells were exposed to H_2O_2, and then treated with extracts RE and RW at concentrations of 50 µg/mL for 24h. Following DCF staining of the cells, the intracellular ROS production was both quantified by spectrofluorimeter assay. Values are reported as % of control and are mean ± S.D from a least three independent experiments. Significant differences are denoted by symbols: C *vs* H_2O_2 insulted cells *$p \leq 0.05$ and H_2O_2 insulted cells *vs* extracts, #### $p \leq 0.0001$.

Figure 5

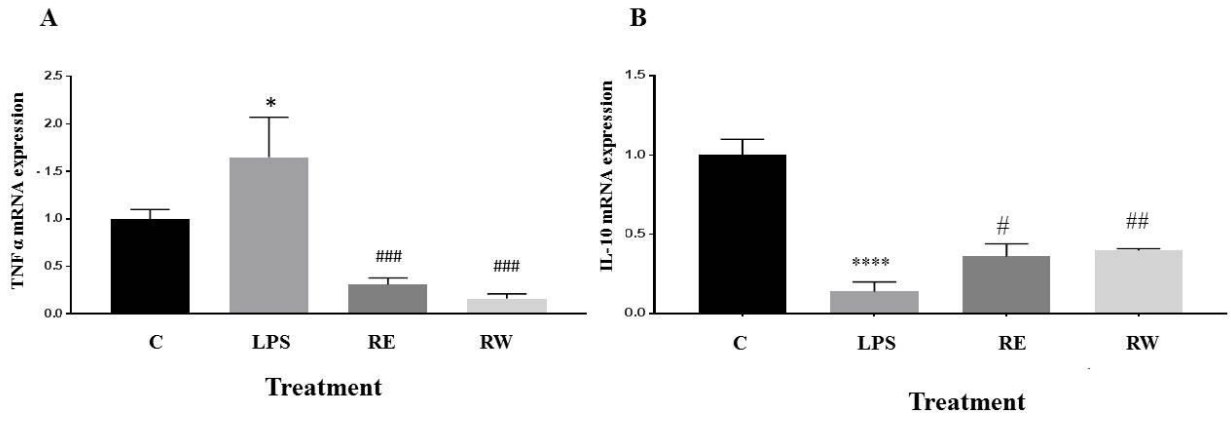

Figure 5. The effect *R. coriaria* extracts on expression of TNFα (A) and IL-10 (B). Cells were treated with 1µg/ml of LPS. Following LPS treatment, cells were treated with either of the two extracts. mRNA levels were determined by qRT-PCR as described in the materials and methods. Cells were counted in triplicate measurements in PCR plate. Values are mean ± S.D from a least three independent experiments. Significant differences are denoted by symbols: C *vs* LPS insulted cells ****p≤0.0001, *p≤0.05 and LPS insulted cells *vs* extracts, ### p≤0.001, ##p≤0.01, #p≤0.05.

Figure 6

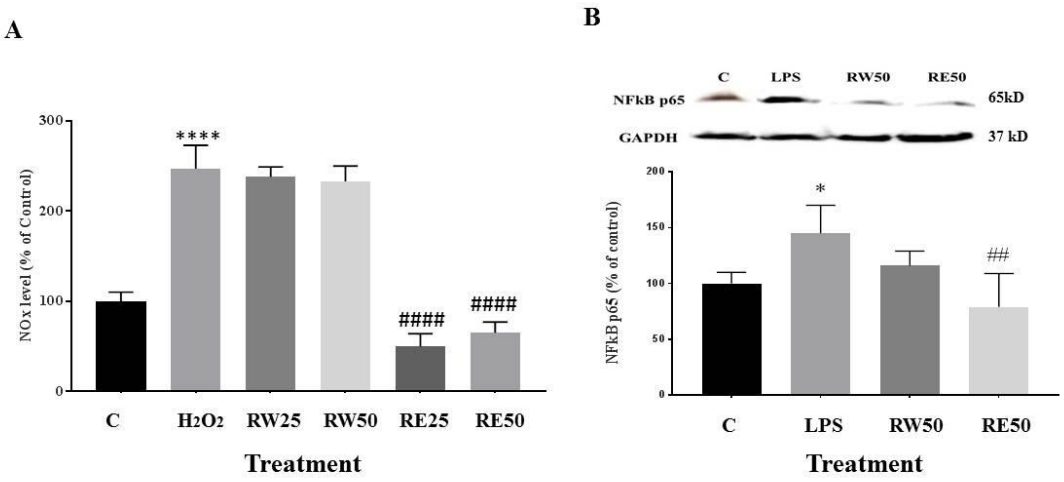

Figure 6. Effect of *R. coriaria* extracts on oxidative stress-induced inflammation in BV-2 cells. (A). BV-2 cells were treated with H_2O_2 and then were treated with both extracts at the tow different concentration (25 and 50 µg/ml) for 24h, Nitric oxide (NO) production was quantified in the medium of BV-2 cells as µmol $NaNO_2$/mg sample protein by Griess reaction. data are expressed as percentage values with respect to controls. Values are mean ± S.D from a least three independent experiments. Significant differences are denoted by symbols: C *vs* H_2O_2 insulted cells ****$p \leq 0.0001$ and H_2O_2 insulted cells *vs* different concentration of extracts, #### $p \leq 0.001$, **(B)** BV-2 cells were treated with LPS and then were treated with both extracts at concentration of 50 µg/ml for 24h, densitometry analysis of NF-kB/p65 was evaluated by Western blotting; GAPDH was the housekeeping gene for normalization; Values are mean ± S.D from a least three independent experiments. Significant differences are denoted by symbols: C *vs* LPS insulted cells *$p \leq 0.05$ and LPS insulted cells *vs* extracts, ## $p \leq 0.01$.

➢ CHAPTER 4

Protective Effects of Extracts from *Ephedra foeminea* Forssk fruits against Oxidative Injury in Human Endothelial Cells

Accepted in *Journal of Ethnopharmacology*

ABSTRACT

Ethnopharmacological relevance: *Ephedra foeminea* is a member of the *Ephedraceae* family which is widespread in the eastern Mediterranean area. In Lebanon, *Ephedra* is a popular remedy in traditional medicine to prevent and/or counteract many stress oxidative-related diseases like inflammation and bacterial infections. ***Aim of the study:*** Oxidative stress leads to endothelial cell dysfunction, and is a major factor contributing to etiology of atherosclerosis and related diseases. In this study, we investigated the antioxidant and cytoprotective potential of extracts from *E. foeminea* fruits on human endothelial cells exposed to hydrogen peroxide to mimic *in vitro* vascular endothelium dysfunction. ***Materials and Methods:*** Different extracts of *E. foeminea* fruits were prepared using pure ethanol (EE), methanol/water mixture (EMW), pure hexane (Ehex) or ethyl acetate/water (Epoly) as extraction solvents. The phenolome profile of each extract was characterized using High Performance Liquid Chromatography-Mass Spectrometry (HPLC/MS) analysis. Total phenolic and flavonoid content, and radical scavenging properties of the extracts were assessed spectrophotometrically. Then, the effects on human endothelial cells HECV were evaluated. ***Results:*** Epoly extract showed the highest phenol and flavonoid content, and the highest radical scavenging capacity. On H_2O_2-insulted HECV cells Epoly was able: *(i)* to counteract the ROS/RNS production and lipid peroxidation; *(ii)* to rescue the ROS-dependent decrease in the mitochondrial membrane potentiall; *(iii)* to counteract the apoptosis induction; *(iv)* to restore endothelial cell viability and migration. ***Conclusions:*** The findings indicated that the polyphenol-enriched extract from *E. foeminea* frutits is endowed with *in vitro* anti-oxidant and anti-apoptotic effects and might be used as nutraceutical for treating radical-related endothelium dysfunction and inflammation.

Introduction

Oxidative stress is a condition caused by an imbalance between production and degradation of oxygen reactive species (ROS), which has been implicated in the pathogenesis of numerous diseases such as metabolic disorders, degenerative diseases and atherosclerosis [Di Meo et al., 2016]. Under physiological conditions, maintaining a low content of intracellular ROS is essential for body health, and the ROS level is strictly regulated by antioxidants, which can be either generated endogenously or externally supplemented [Liu et al., 2018].

Chronic diseases such as diabetes, metabolic syndrome, and atherosclerosis are complex inflammatory illnesses that involve numerous physiological systems. Vascular endothelium is a physiological barrier protecting against blood cell adhesion, maintaining vascular tonus, and inhibiting proliferation of smooth muscle cells. Excess ROS accumulation and consequent oxidative stress in vascular endothelium lead to cell dysfunction and apoptosis [Salisbury and Bronas, 2015; Incalza et al., 2018]. Apoptotic endothelial cells exert pro-coagulant effects and promote adhesion of platelets thus contributing to atherosclerosis and thrombosis [Bombeli et al., 1999]. Therefore, protection of endothelial cells from oxidative injury and apoptosis may be a beneficial strategy for prevention/treatment of vascular diseases.

Nowadays, an increasing number of people seek for preventive therapies and long term treatments using nutraceuticals. Currently, more than 20,000 plant species are considered as potential reservoirs for new drugs [Amor et al., 2009]. In the last years, nutraceuticals have gained attention as possible treatment of diseases like obesity, diabetes and coronary diseases.

The Eastern Mediterranean regions are rich in medicinal plants and people have long histories on their use in traditional medicinal. In Lebanon, *Ephedra foemina* (Alanda in traditional Arab) is a popular herbal "wonder drug"; it belongs to *Ephedra*, a genus of *Ephedraceae* family [Mighri et al. 2017]. The activities of *Ephedra* include antioxidant [Okawa et al. 2001], anti-asthmatic [Liu and Luo 2007], anti-inflammatory [Aoki K et al., 2005], anti-microbial [Zang et al. 2013], anti-proliferative [Danciu et al. 2018], hypoglycemic [Xiu et al. 2001; Ben Lamine et al. 2019] and weight reduction [Roh et al. 2017; Lim et al. 2018; Lee et al., 2019] effects. *Ephedra* plants are rich in bioactive compounds including polyphenols and alkaloids [Zang et al. 2018, Ziani et al. 2019]. The best known bioactive compounds of *Ephedra* are ephedrine-type alkaloids (ephedrines), whose content varies from species to species [Ibragic & Sofić, 2015]. The side

effects of ephedrine-type alkaloids are important as they act on the central nervous system, blood pressure, dilates and pulse rate.

Interestingly, the species *E. foeminea* is completely lacking of ephedrine-type alkaloids [Caveney et al., 2001], but little is still known about its bioactive constituents. Therefore, extracts from *E. foeminea* lacking of ephedrine-type alkaloids are of interest as possible nutraceuticals. In Lebanon and in the Mediterranean area, the use of *E. foeminea* is well documented (Ali-shtayeh et al., 2016; Labidi et al., 2020); people can collect the whole plant directly or buy it from herbalist shops called "Dabbous" (Liozzo et al. 2008). Ben-Arye et al. (Ben-Arye et al., 2016), conducted an extensive search about traditional use of *E. foeminea* using a number of textbooks such as Medieval Arabic and Hebrew medical literature, *Materia Medica* and pharmacopeia in Arabic, Hebrew and Judeo-Arabic. Many reports conclude that the plant is used in traditional Arab medicine as a treatment for anxiety and skin rash, besides, the whole plant has bronchodilator properties and it is used for the treatment of various respiratory problems, mainly against bronchial asthma (Philips, 1958).

The present study aimed at preparing, characterizing and investigating four different extracts from *E. foeminea* fruits collected in South Lebanon. The extracts were prepared using different solvents in order to optimize the extraction of different polyphenol groups. The phytochemical composition of each extract and the radical scavenging ability were assessed. The Epoly extract being prepared using ethyl acetate/water as solvent showed the highest polyphenol content and scavenging ability. Then, the antioxidant, anti-inflammatory and cytoprotective activities of the four extracts were assessed on HECV endothelial cells upon an oxidative insult to mimic endothelium dysfunction being associated to atherosclerosis. Also in this case, Epoly showed the best potential as antioxidant and cytoprotective agent. Our findings indicate that Epoly is endowed with significant *in vitro* anti-inflammatory and cytoprotective effects, thus representing a good nutraceutical candidate.

Materials and Methods

2.1 Plant material and extraction

Fresh fruit material of *Ephedra Foeminea* Forssk (http://www.theplantlist.org/tpl1.1/record/kew-332925), synonym *Ephedra campylopoda*, was collected from *Maarakeh* (South Lebanon), 280 m above sea level (Coordination 33.264810' N and 35.307050' E), in the month of January 2018. The plant was identified by Prof Mario Mariotti (DSTAV, University of Genoa- Italy) and by Dr. George Tohme, Professor of Taxonomy and President of the National Council for Scientific Research (CNRS), Beirut, Lebanon, a voucher specimen (E2.1/4) has been deposited in the Herbarium of the Faculty of Science, Lebanese University, Hadath, Beirut, Lebanon.

The fruits of *E. foeminea* were shade dried for three weeks at room temperature. Then, the grinded materials were subjected to solvent extraction with 100% ethanol, 100% hexane or 50:50 % methanol:water, alternatively, using standard procedures [Ray et al. 2018]. In details, 25 grams of dried material were dissolved in 300 ml of each solvent and placed on stirrer overnight at room temperature. At the end, solvents were evaporated in a Rotavac vario power unit (Heidolph Instruments, Schwabach, Germany), and then freeze-dried in Alpha 1-4 LD plus lyophilizer (CHRIST, Osterode am Harz, Germany). Then, the dry fractions were stored at -20°C until use. A fourth extraction procedure was used to optimize the extraction of water-soluble polyphenols [Abu-Reidah, 2015 and Babbar 2014]. Briefly, 15 grams of powdered *E. foeminea* fruits were wetted with 5 mL of NH4OH (25% m/m) and extracted with 300 mL of ethyl acetate for 72h at room temperature. The extract was filtered and the solvent was evaporated in a rotary evaporator Rotavac vario power unit (Heidolph Instruments, Schwabach, Germany) under reduced pressure at 40°C. The residue was then dissolved in ddH_2O, and then further extracted with hexane to get rid of the non-polar fraction that could be extracted by ethyl acetate. At the end, the extract was freeze-dried in Alpha 1-4 LD plus lyophilizer (CHRIST, Osterode am Harz, Germany) to obtain water soluble polyphenolic-enriched extract.

2.2 HPLC-MS analysis

High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS/MS) was performed using an Agilent 1100 HPLC-MSD Ion Trap XCT system, equipped with an electrospray ion source (HPLC-ESI-MS) (Agilent Technologies, Santa Clara, CA, USA). Separation of extracts was performed on a Jupiter C18 column 1 mm × 150 mm with 3.5 μm particle size (Phenomenex, USA). As eluents we used water (eluent A) and MeOH (eluent B),

both added with 0.1% formic acid. The gradient employed was: 15% eluent B for 5 min, linear to 100% eluent B in 35 min, and finally hold at 100% eluent B for another 5 min. The flow rate was set to 50 µL/min with a column temperature of 30°C. The injection volume was 8 µL. Ions were detected in the positive and negative ion mode, in the 100–800*m*/*z* range, and ion charged control with a target ion value of 100,000 and an accumulation time of 300 ms. A capillary voltage of 3300 V, nebulizer pressure of 20 psi, drying gas of 8 L/min, dry temperature of 325°C, and 2 rolling averages (averages: 5) were the parameters set for the MS detection. MS/MS analysis was conducted using an amplitude optimized time by time for each compound. From the chromatograms, the percentage of PC for each extract was calculated on the basis of the peak area [Khalil et al., 2019].

2.3 Total Phenol Quantification

Total phenol content (TPC) was determined for each extract using the Folin–Ciocalteu method [Singleton, Rossi, 1965]. Briefly, 100 µl aliquots of each extract (1 mg/ml) were incubated with 0.5 ml of 10% (w/v) *Folin–Ciocalteau* reagent. After 5 min, 1.5 ml of Na_2CO_3 (2% w/v) were added and incubated in the dark at room temperature for 30 min. The absorbance was measured at 760 nm using an U-2900 UV-Vis spectrophotometer 200 V (Hitachi High-Technologies, Japan) against a blank w/o extract. The results were derived from a calibration curve of gallic acid (0–250 □g/mL) prepared from a stock solution (1 mg/mL in ethanol) and expressed in Gallic Acid Equivalents (GAE) per gram dry extract weight.

2.4 Total Flavonoid Quantification

Total flavonoid content (TFC) was determined for each extract using aluminium chloride ($AlCl_3$) colorimetric method [Arvouet-Grand et al., 1994]. Briefly, 1 ml aliquot of each extract (1 mg/ml) was mixed with 0.2 ml of 10% (w/v) methanolic $AlCl_3$ solution, 0.2 mL (1 M) potassium acetate and 5.6 mL distilled water. After incubation at room temperature in darkness for 30 min, the absorbance was measured at 415 nm using UV-Vis spectrophotometer. The results were derived from a calibration curve of quercetin (0-200 µg/mL) prepared from a stock solution (5 mg/mL in methanol). The results were expressed in mg of Quercetin Equivalent (QE) per g of dry weight of extract powders.

2.5 DPPH radical scavenging assay

The radical scavenging activity of each extract was measured using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method [Srinivasan et al., 2007]. Briefly, in a 96-multiwell plate, 50 µL aliquot of extract (1–5000 □g/mL) was added to 200 µL of DPPH (0.1 mM in methanol) solution. The mixtures were incubated at 37°C in darkness for 20 min, and then absorbance was measured at 490 nm using a microplate reader (FLUOstar Optima, BMG Labtech) against an equal amount of DPPH solution as a blank. The percentage of DPPH scavenging was estimated using the equation:

$$\% \text{ scavenging activity} = [(\text{Abs control} - \text{Abs sample})]/ (\text{Abs control})] \times 100$$

Values are expressed as IC_{50}, defined as the concentration of the sample required to cause a 50% decrease in initial DPPH, as well as µmoles Trolox equivalent (TE)/L.

2.6 ABTS radical cation scavenging assay

The radical cation scavenging activity of each extract was performed using the 2-2′-azino-bis(3-ethylbenzo-thiazoline-6-sulphonate) diammonium salt (ABTS) method [Re et al., 1999]. Briefly, in a 96-multiwell plate 50 µL aliquot of each extract (1–5000 □g/mL) was added to 200 µL of ABTS solution (5 mM). ATBS solution was prepared by oxidizing ABTS with MnO_2 in distilled water for 30 min in the dark, and then the solution was filtered through filter paper. After incubation at room temperature for 20 min, the absorbance of each solution was determined at 734 nm against an equal amount of ABTS solution as a blank using a microplate reader (FLUOstar Optima, BMG Labtech). The percentage of ABTS scavenging was estimated using the equation:

$$\% \text{ scavenging activity} = [(\text{Abs control} - \text{Abs sample})]/ (\text{Abs control})] \times 100$$

Values are expressed as IC_{50}, defined as the concentration of the sample required to cause a 50% decrease in initial ABTS concentration, as well as µmoles Trolox equivalent (TE)/L.

2.7 Ferric Reducing Antioxidant Power (FRAP) Assay

The reducing power was evaluated according to the Ferric Reducing Antioxidant Power (FRAP) assay [Firuzi et al., 2005]. Briefly, in a 96-multiwell plate, 25 µL aliquot of each extract (1–5000 □g/mL), or of standard Trolox, (0- 250□g/mL), was added to 175 µL of FRAP working solution containing 300 mmoL/L acetate buffer (pH 3.6), 20 mmoL/L ferric chloride and 10 mmoL/L TPTZ (2,4,6-tri (2-Pyridyl) –S-triazine) made up in 40 mmoL/L hydrochloric acid. All three solutions were mixed together in the ratio 10:1:1 (v:v:v), respectively. The mixtures were incubated at 37°C for 30 min in the dark, and then the absorbance of each solution was determined at 593 nm against FRAP solution and methanol as a blank, using a microplate reader (FLUOstar Optima, BMG Labtech, Ortenberg, Deutschland).

2.8 Cell culture and treatments

HECV cells (Cell Bank and Culture-GMP-IST-Genoa, Italy), an human endothelial cell line isolated from umbilical vein, were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium High Glucose (D-MEM) supplemented with L-Glutamine and 10% FBS. For treatments, cells were grown until 80% confluence. To induce oxidative stress, HECV cells were incubated for 24 h with H_2O_2 (30 µM). Thereafter, cells were incubated for 24h in fresh medium containing the extracts at different concentrations (0, 25 and 50 µg/ml). For each experiment, treatment was performed in quadruplicates.

2.9 Cell viability assay

HECV cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method according to Hussain et al, 1993. Stock solutions (20 mg/mL) were prepared in dimethyl sulfoxide (DMSO) for the ethanolic and methanolic-Aqueaous extracts and sterile double distilled water ddH_2O for the water-soluble polyphenolic extract. For analysis, cells were seeded in 96-well plate (10^4 cells per well), in complete medium. After 24 h (confluency ≈ 75%), HECV cells were incubated for 24 h with H_2O_2 (30 µM). Thereafter, cells were incubated for 24h with extracts at different concentrations (0, 10, 25 and 50 µg/ml). At the indicated times, 20 µL of MTT reagent (5.0 mg/mL) was added and incubated at 37°C for 3 h. Then, the unreacted MTT dye was removed by aspiration and 100 µL of acidified isopropanol was added to solubilize purple formazan crystals within metabolically active cells. The absorbance of sample was recorded at 570 nm by multidetection microplate reader (Biotek

Instrument, Winooski, VT, USA). Data are expressed as percentage of cell viability with respect to control. For each experiment, treatment was performed in quadruplicates.

The inhibitory rate was calculated by the following formula:

$$\text{Inhibitory rate (\%)} = (1- At/Ac) \times 100$$

Where *At* and *Ac* were the absorbance values of the treatment and control wells, respectively.

2.10 ROS production

The oxidation of the cell-permeant 2'-7' dichlorofluorescin diacetate (DCF-DA, Fluka, Germany) to 2'-7'dichlorofluorescein (DCF) allowed to quantify *in situ* the production of H_2O_2 and other ROS [Halliwell and Whiteman, 2004]. Stock solution of DCF-DA (10 mM in DMSO) was prepared and stored at -20°C in the dark. At the end of treatment cells were scraped and gently spun down (600xg for 10 min at 4°C). After washing, cells were loaded with 10 mM DCF-DA in PBS for 30 min at 37°C in the dark. Then, cells were centrifuged, suspended in PBS and the fluorescence was measured fluorometrically (□ex=495 nm; □em=525 nm). All measurements were performed using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies Italia SpA, Milan, Italy) at 25°C using a water-thermostated cuvette holder. The stained cells were also observed by Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany). Image analysis was performed using ImagJ software (http://imagej.nih.gov/ij/). In each image field, the total number of pixels was quantified on a gray scale, the average intensity (mean pixel value for each cell) was calculated and expressed as mean±SD of average intensity of the total of 40 cells area in each samples.

2.11 Lipid peroxidation

Lipid peroxidation was determined spectrophotometrically through the thiobarbituric acid reactive substances (TBARS) assay which is based on the reaction of malondialdehyde (MDA; 1,1,3,3-tetramethoxypropane) with thiobarbituric acid (TBA) [Iguchi et al. 1993]. Briefly, 1 vol. of cell suspension was incubated for 45 min at 95°C with 2 vol. of TBA solution (0.375% TBA, 15% trichloroacetic acid, 0.25 N HCl). Then, 1 vol. of N-butanol was added and the organic

phase was read at 532 nm in a UV-VIS spectrophotometer at 25°C using Peltier-thermostated cuvette holder. The MDA level was expressed as pmol MDA/mL/mg protein.

2.12 Nitrite/Nitrate (NOx) Levels

NO production was measured by spectrophotometric measurement of the end products, nitrites and nitrates, using the Griess reaction [Green et al. 1982]. After treatments, nitrite accumulation (μmol NaNO2/mg sample protein) was calculated against a standard curve of sodium nitrite (NaNO2). All spectrophotometric analyses were carried out at 25°C recording absorbance at 540 nm.

2.13 Micronucleus test

The number of micronuclei in HECV cells were measured using the fluorescent nuclear dye, 4′-6-diamidino-2-phenylindole (DAPI) following Valovicova et al. (2009). About 5×104 cells were seeded on coverslips in 6-wells plates. Cells were treated for 24 h with H_2O_2 and then cells were treated with the polyphenolic extract Epoly. After 24 hours of treatment cells were, washed with PBS and fixed with 4% paraformaldehyde for 20 min. Then, cells were stained with DAPI (1 μg/mL in PBS) for 10 min. Coverslips were mounted onto the slides using mounting media. Morphologic changes in apoptotic cells were observed under a fluorescence microscope Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany).

2.14 Mitochondrial membrane potential

The mitochondrial membrane potential (ΔΨm) was assessed using the fluorescent probe tetramethylrhodamine methyl ester perchlorate (TMRM), a cationic lipophilic, non-toxic fluorescent compound. After washing, cells were loaded with 0.2 μM TMRM solution in PBS for 30 min at 37°C in the dark. Then, cells were centrifuged, suspended in PBS and fluorescence was measured fluorometrically (ex=549 nm; em=575 nm). All measurements were performed using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies) at 25°C using a water-thermostated cuvette holder. The stained cells samples were also observed by fluorescence microscopy. For each sample, fluorescent intensities were analysed using ImageJ software. In each image field, the total number of pixels was quantified on a gray scale, and the

average intensity (mean pixel value for each cell) was calculated and expressed as mean±SD of average intensity of the total number of cells area in each samples.

2.15 Wound Healing assay

The migration of HECV was evaluated using the wound healing assay [Rodriguez et al. 2005]. The cells were seeded on 35×10 mm tissue culture dishes and incubated until confluence was reached, the cell monolayer was scraped with a p100 pipet tip making two crossing straight lines to create a ‘‘scratch’’. Then, five views on the cross were photographed by an inverted Olympus IX53 microscope (Olympus, Milan, Italy) and representative images were captured with a CCD UC30 camera and a digital image acquisition software (cellSens Entry). After scratching, cells were washed with PBS and then incubated with fresh medium in the absence or presence of polyphenolic extract at 25 or 50 μg/mL. Set of images were acquired at 0, 6 and 24 h. To determine the migration of HECV, the images were analysed using ImageJ free software (http://imagej.nih.gov/ij/). Percentage of the closed area was measured and compared with the value obtained before treatment. An increase of the percentage of closed area indicated the migration of cells. Data are means ± S.D. of at least three independent experiments.

2.16 Protein quantification

The protein content was determined by the Bradford assay using bovine serum albumin (BSA) as a standard [Bradford, 1976].

2.17 Statistical analysis

All results were expressed as mean ± SD of at least three independent experiments. GraphPad Prism 7.0 software was used for statistical evaluation. Comparisons between different conditions were performed using one-way ANOVA with Tukey’s post-test (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

3.1 Phenolome characterization of the four *E. foeminea* extracts

Different extraction procedures supplied extracts being enriched in different bioactive compounds depending on their polarity [Wang and Weller 2006]. Typically, phenolic compounds are soluble in polar solvents due to the presence of hydroxyl groups, and pure ethanol and methanol/water mixture (50:50 V/V) were employed as extraction solvents, and we referred to these extracts as the Ethanol Extract (EE) and the Methanol-Water Extract (EMW), respectively. Pure hexane was used for extraction of non-polar compounds like sterols and the extract was named Ehex. The fourth extract was obtained using ethyl acetate and water as solvents in order to optimize the extraction of polyphenols and we named it the polyphenol-enriched extract (Epoly).

The phenolome profiling of the four extracts was characterized by HPLC-DAD-MS/MS. Table 1 summarizes all the compounds identified in each extract, with their MS^2 fragmentation pattern in negative and positive mode, respectively. In total sixty nine PC were identified in the extracts, with differences among extracts depending on the elution solvent. In all four extracts, we identified twenty five flavonoid glycosides (with one or two sugar moiety) and they include flavonols (Quercitin, Myricetin, Isorahmnetin), flavones (Apegnin) and anthocyanins (Cyanidin, Malvidin). Quercetin rhamoniside and cyanidin-3-O-rhamnoside were the major components in all extracts, except in EHex, and were more abundant in Epoly than in EE and EMW. Interestingly, quercetin rhamoniside previously reported in *Ephedra alata* [Zhang et al. 2018], here was identified also in *E. foeminea*. EE, EMW and Epoly extracts contained fifteen hydroxycinnamic acids which are mostly derivatives of coumaric and ferulic acid. Conversely, EHex was rich in the sterols β-sitosterol, citrostadienol glucoside and sitostanol glucoside which were the most abundant component.

3.2 Polyphenol content and radical scavenging activity of the *E. foeminea* extracts

The four extracts were characterized for their total phenol and flavonoid content (TPC and TFC), which are summarized in Table 2. Significant differences in TPC and TFC could be observed among the extracts (Fig. 1A). Epoly showed the highest phenol content (31.5 mg GAE/g dry extract) followed by EMW (with 18.6 mg GAE/g dry extract), EE (with 14.8 mg GAE/g extract), and Ehex with the lowest TPC (1.5 ± mg GAE/g extract /g). A similar trend was observed for

TFC with Epoly showing the highest flavonoid content (14.5 mg QE/ g dry) followed by EMW (10 mg QE/g dry extract), and EE (8 mg QE/g extract. Ehex showed no-detectable amounts of flavonoids.

The *in vitro* scavenging activity of the extracts was assessed by using three spectrophotometric assays: ABTS, DPPH, and FRAP. The values expressed as Trolox equivalents are reported in Table 2 and compared in figure 1B. As expected Epoly showed the highest scavenging potential with rather high DPPH, ABTS and FRAB values (0.286, 14.28 and 0.37 mmol TE/L, respectively). Both EMW and EE showed a moderate scavenging activity. EMW extract showed DPPH, ABTS and FRAB values of 0.11, 3.55 and 0.15 mmol TE/L, respectively. EE extract showed ever lower DPPH, ABTS and FRAB values (0.097, 1.95 and 0.13 mmol TE/L, respectively). No radical scavenging activity was detectable for Ehex.

We tested the possible correlation between TPC, TFC and the three markers of radical scavenging activity (Fig. 1C). For all extracts we found an excellent correlation between TPC and TFC (r^2=0.99; $p<0.01$), and a good correlation between TPC and the parameters DPPH (r^2=0.97; $p<0.05$) and FRAP (r^2=0.97; $p<0.05$). Instead, the correlation between TFC and the scavenging activity parameters was high but non-significant (DPPH, r^2=0.93; ABTS, r^2=0.84; FRAP, r^2=0.92). As expected, the scavenging activities determined by the three different methods correlated significantly ($p<0.05$) between each other: DPPH/ABTS (r^2=0.98); DPPH/FRAP (r^2=0.99); ABTS/FRAP (r^2 = 0.98).

3.3 Cytoprotective effects of the *E. foeminea* extracts on oxidative injured endothelial cells

Before using the extracts for experiments on cells, we tested for their possible cytotoxicity on HECV cells. At all the tested concentrations no cytotoxic effect was observed for each extract (data not shown). We excluded Ehex from the further studies as it showed no radical scavenging activity at all.

We verified the possible cytoprotective activity of the extracts on dysfunctional endothelial cells. To mimic an oxidative insult HECV cells we exposed to 30 μM H_2O_2 for 24h, and to the extract (doses from 10 to 50 μg/mL) for 24 h. The protective effects of *E. foeminea* extracts were assessed at three different levels: (*i)* to prevent the H_2O_2-induced cytotoxicity (pre-treatment with extracts followed by exposure to H_2O_2); (*ii)* to protect by the H_2O_2-induced cytotoxicity (co-

treatments of cells with extracts and H_2O_2); *(iii)* to rescue the H_2O_2-induced cytotoxicity (pre-treatment with H_2O_2 followed by treatment with extracts.

Exposure of HECV cells to H_2O_2 for 24h led to a significant decrease in cell viability (about -30%; $p \leq 0.01$) compared with control (Fig. 2). The pre-treatments of cells with the Epoly extract before the oxidative insult did not protect cells against H_2O_2 cytotoxicity (Fig. 2A). Also the simultaneous treatment with Epoly + H_2O_2 led to non-significant protective effect (Fig. 2B). By contrast, the H_2O_2-induced cytotoxicity was significantly alleviated when Epoly was added after H_2O_2-insult leading to a significant restore of cell viability (+16 % for Epoly 25 μg/mL, and +18 % for Epoly 50 μg/mL; $p \leq 0.05$) (Fig. 2C). The other extracts EE and EMW did not show any protective effect against oxidative insult in all the experimental conditions (data not shown). Therefore, Epoly resulted the most effective extract *in vitro* and the most protective one *in vivo*.

3.4 Antioxidant effects of the *E. foeminea* extracts on oxidative injured endothelial cells

Because Epoly was effective when used to rescue the H_2O_2-induced cytotoxicity (pre-treatment with H_2O_2 followed by treatment with Epoly) we further investigated if this beneficial activity was mediated by effects on ROS production and lipid peroxidation.

The intracellular ROS production was quantified by fluorimetric analyses of DCF-stained HECV cells exposed to 30 μM H_2O_2 for 24h, then to the extracts for 24 h (doses of 25 and 50 μg/mL). Exposure of endothelial cells to H_2O_2 resulted in a significant increase in DCF fluorescence (+44%; $p \leq 0.001$). Exposing the H_2O_2-insulted cells to Epoly led to a significant decrease in the ROS level (-53% and -47% for 25 μg/mL and 50 μg/mL, respectively; $p \leq 0.01$). Also EE at the highest dose (50 μg/mL) reduced the ROS level of about -45% ($p \leq 0.05$), while EMW had no effects (Fig. 3A). The intracellular levels of ROS were also visualized *in situ* by fluorescence microscopy of DCF-stained cells. In line with fluorimetric analysis, higher and diffuse DCF fluorescence was observed in H_2O_2-insulted cells (+38% vs control; $p \leq 0.01$), and it was reduced after treatment with Epoly (25 and 50 μg/mL) of about -27% and -33% ($p \leq 0.05$), respectively, with respect to insulted cells (Fig. 3B). No differences could be appreciated for EE and EMW (data not shown).

The ROS-induced lipid peroxidation, a classical marker for oxidative stress, was assessed by TBARS assay (Fig. 3C). H_2O_2–insulted cells showed a marked increase in MDA level (+56% with respect to controls; $p \leq 0.0001$) and Epoly was able to rescue this effect leading to a decrease in MDA level of about -39% ($p \leq 0.01$) for both doses. Both EE and EMW showed no-significant effects on MDA level.

3.5 Effects of the *E. foeminea* extracts on counteracting NO production and apoptosis in oxidative injured endothelial cells

On light of the results above described, we focused on Epoly for the further analyses. The NO production, a classical marker for inflammation and vascular diseases, was increased in HECV cells injured with hydrogen peroxide (+35%; $p \leq 0.01$) with respect of control (Fig. 4A). At both concentrations (25 μg/mL and 50 μg/mL) Epoly was able to counteract the stimulation of NO release compared to H_2O_2-insulted cells (about -45% and 49 %, respectively; $p \leq 0.001$).

Treatment with Epoly was also able to counteract the cell shrinking induced by oxidative insult. Figure 4B shows a panel of representative optical micrographs of HECV cells in different conditions. While the untreated HECV cells (control) showed classical endothelial morphology and were adherent and well spread to substrate, exposure to H_2O_2 led to a cell shrinking, which is a classical apoptosis-like phenotype, with an increase in intracellular granular material and cell rounding. Upon treatment with Epoly (25 or 50 μg/mL), the cells recovered a morphology rather similar to that of control cells.

DAPI staining in H_2O_2-insulted cells revealed an increased number of nuclei with chromatin condensation, apoptotic bodies and micronuclei which are characteristic morphological changes of early-apoptosis phenotype (Fig. 4B). Treatment with Epoly at both concentrations (25 and 50 μg/mL) significantly rescued the H_2O_2-induced apoptotic phenotype. On the other hand, the control showed no apoptotic cells (less than 10% of total cells).

Apoptosis is linked to alteration in mitochondrial function and integrity that we assessed by measuring the inner membrane potential ($\Delta\Psi$m) using the fluorescent probe TMRE. By fluorimetric analysis of cell suspension, we observed a slight increase (+16%) in $\Delta\Psi$m after exposure to H_2O_2 compared to controls (Fig. 5A). The $\Delta\Psi$m was increased markedly by Epoly

treatment at both 25 μg/mL and 50 μg/mL concentrations (+46% and +52%, respectively; $p \leq 0.05$) with respect to control. The cells were also observed by fluorescence microscopy giving results consistent with those obtained with the fluorimetric analysis (Fig. 5B). Exposure to H_2O_2 led to a non-significate increase in TMRE fluorescence compared to controls, by contrast, the TMRE signal was increased strongly by Epoly treatment (25 μg/mL and 50 μg/mL concentrations) of about +160% and +166% ($p \leq 0.05$) respectively.

3.6 Effects of the *E. Foeminea* extract Epoly on wound repair of endothelial cells

The modulation of wound healing ability of HECV cells was assessed by T-scratch assay. When the scratched cell layer was incubated for 6 and 24 h in the presence of Epoly (at 25 or 50 μg/mL) we observed an average acceleration of the wound repair compared to control, and the effect was significant at a the longest time (24 h) (Fig. 6A). While the control cells reduced the wound width to 34% after 24h compared to time 0 (refereed as 100%), both concentrations of Epoly significantly stimulated cell migration resulting in a wound width smaller than that of control at the same time. After 24 h the wound width was reduced up to of 25% and 23% ($p<0.05$) in the presence of Epoly 25 μg/mL and 50 μg/mL, respectively (Fig. 6B).

Discussion AND CONCLUSION

In traditional medicine, *Ephedra* has mainly been used to treat respiratory diseases such as asthma, but only recently *Ephedra* plants have been tested for weight reduction properties [Roh et al. 2017; Lim et al. 2018; Lee et al., 2019]. Here, we found the potential of *E. foeminea* extracts as source of natural antioxidants protecting against free radical-induced damage in endothelial cells. Indeed, oxidative imbalance is the primary cause of endothelial dysfunction leading to vascular damage in both metabolic and atherosclerotic diseases [Incalza et al., 2018].

In this context, we have prepared four different extracts from *Ephedra foeminea* fruits using pure ethanol (EE), methanol/water mixture (EMW), pure hexane (Ehex) or ethyl acetate/water (Epoly) as solvent. The last one was optimized to supply a water-soluble polyphenol-enriched extract. Indeed, poor aqueous solubility is the major problem encountered by pharmaceutical industry for most phytochemicals [Savjani et al. 2012]. The phenolome profile of all extracts

contained mainly flavonoids (in glycosylated form) and hydroxycinnamic acid derivatives. The quercetin-3-O-rhamoniside and the cyanidin-3-O-rhamnoside are the most abundant components in all extracts. Quercetin is widely diffuse in *Ephedra* plants and its derivatives have been proven to have a spread of pharmacological properties [Kawada el al., 1998; Hong et al., 2013]. Also hydroxycinnamic acids are common in many species of *Ephedra*, and here they were identified also in *E foeminea* [Spinola et al. 2015]. Interestingly, hydroxycinnaoyl-gentioboses and cinnamoyl-amino acid conjugates were reported for the first time in the *Ephedreacea* family.

Among the four extracts, Epoly showed the highest phenolic and flavonoid content, that resulted in the highest *in vitro* radical scavenging activity. In fact, we found a very high correlation (r^2>0.97) among TPC, TFC and the antioxidant parameters that we tested in this work by DPPH, ABTS and FRAP analyses.

HECV cells exposed to hydrogen peroxide can mimic endothelium dysfunction and inflammation occurring *in vivo* during atherosclerosis [Vergani et al. 2017; Khalil et al. 2019]. As the scavenging of radicals occurs typically in very short times (minutes), but the activation of intracellular antioxidant mechanisms occur in longer times (hours), the treatments were carried for 24 h. Exposure of HECV cells to 30 μM H_2O_2 for 24 h was employed as moderate oxidative insult, according with previous studies [Khalil et al. 2019]. The oxidative damage and the rescue played by extracts, Epoly especially, were measured at different levels. At first, we assessed the intracellular ROS and NO production, and the level of lipid peroxidation and apoptosis being associated with the radical over-production. In H_2O_2-insulted cells we observed increased levels of ROS and NO, and consequent increase in MDA production and apoptotic phenotype. Epoly (25 and 50 μg/mL) led to a significant rescue of oxidative stress and cell dysfunction by decreasing ROS production, MDA level (indicator of lipid peroxidation) and NO release (indicator of endothelium inflammation and oxidative damage).

Epoly was also able to recover cell viability after H_2O_2-insult and protected HECV cells from H_2O_2-induced apoptosis. Indeed, the number of cells characterized by apoptotic nuclear morphology decreased in cells treated with Epoly, in parallel with the rescue of cell viability.

Interestingly, Epoly acts at mitochondrial level by increasing the mitochondrial membrane potential ($\Delta\Psi$m) which was reduced in H_2O_2-insulted cells. As mitochondria play an important

role in oxidative stress and antioxidant defense, our findings may suggest that the beneficial effects of Epoly may depend, at least in part, on its action on mitochondria.

Finally, Epoly was able to accelerate *in vitro* the wound repair of a confluent monolayer of scratched HECV cells. This effect might contribute to the repair of endothelium injured during atherosclerosis.

The interest for bioactive products from plants is increasing with the aim of improving the protocols and approaches for treatment of chronic diseases [Bagherniya et al., 2018]. We wish to emphasize as the *Ephedra* genus is one of the most ancient medicinal plants in the Mediterranean area, and the species *E. foeminea* is getting more popularity in traditional medicine in Lebanon. Despite the popularity, little information are available about its phytochemicals constituents. To the best of our knowledge, this work is the first study performed to identify the detailed phytochemical compositions of *E. foeminea* fruits, and to compare the effects of different solvents on the phenolome profile of the extracts. The main finding of our study was demonstrating the beneficial activity of the polyphenolic components of *E. foeminea* on oxidative-injured endothelial cells mimic the endothelium dysfunction during atherosclerosis.

In conclusions, our findings show the therapeutic potential of extracts from *E. foeminea* fruits as source of natural antioxidants exerting beneficial effects against free radical-induced damage in human endothelial cells. Such an effect may be related to a direct radical scavenging activity of polyphenols being present in high amount in the extracts, but also to their direct action on mitochondria resulting in anti-apoptotic effect. The good antioxidant and anti-apoptotic activity of the extracts, in particular of the one prepared using ethyl acetate/water as solvent (Epoly), together with the ability of stimulating the wound repair make Epoly a good candidate as nutraceutical for treating radical-related endothelium dysfunction and inflammation.

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Tables

Table 1: Phytochemical compounds detected in the four extracts from *E. foeminea* L. fruits by using HPLC–DAD/MS in positive and negative ionization modes. Compounds were numbered by their elution order. The extracts were prepared using pure ethanol (EE), methanol/water mixture (EMW), pure hexane (Ehex) or ethyl acetate/water (Epoly) as extraction solvents

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Peak	RT (min)	[M+H]+ (m/z)	[M_H]⁻ (m/z)	MS/MS fragments	Proposed compound	Extracts			
						EE	EMW	EHex	Epoly
1	5.8	328	326	**a** 309.9; 292 **b** 236; 277.8; 164.5	p-Coumaroyl tyrosine	+	+	-	-
2	10.7	514	512	**a** 367.8; 305 **b** 468; 322	Unknown	-	+	-	-
3	10.8		315	**b** 152.7; 134.8; 109	3, 4-Dihydroxybenzoic acid-O-glu	-	-	-	+
4	11.8	367	365	**a** 349;229;331;271;254 **b** 275;203;317;347,161	caffeoyl-N-tryptophan	-	+	-	-
5	12.8		380.5	**b** 362,308,147,290	Unknown	-	+	-	-
6	12.9		543	**a** 497, 475, 351, 407, 437, 296	3,5-Diferuloylquinic acid	-	+	-	-
7	13.1		475	**b** 136.7,441,355,299	Chrysoeriol-uronic acid	-	+	-	-
8	14.7		511	**b** 349; 403; 431; 269; 467; 241	Caffeoyl-N-tryptophan-rhamnoside	-	+	-	-
9	14.8		781.3	**b** 583; 297.8; 376.8; 458.8; 421	Malvidin-3-glu-(epi)catechin A-type linkage	+	-	-	-
10	14.8		791.2	**b** 745; 583; 459; 296.7	Malvidin-3-glu-(epi)catechin derivate	+	-	-	-
11	15.4		395	**b** 332.7, 376.8,215,146,172,305	acetyl caffeoyl quinic acids	+	+	-	-
12	15.4		479	**b** 432.8; 395; 330; 288.8; 461	Unknown	+	-	-	-
13	15.4		463	**b** 417; 395; 293.2	herbacetin 7-O-glucoside	+	-	-	-
14	16.8		501.7	**b** 193,381,337,178,149	feurilic acid derivate	+	+	-	-
15	17		471	**b** 163,380,307,205	cumaric acid derivate	+	+	-	-
16	17		517	**b** 471	feurilic acid derivate	+	+	-	-
17	18.5		723	**b** 534.9; 675		-	-	-	+
18	18.5		551	**b** 164.7; 384.7; 482.8; 414.8	Caffeoyl monotropein hexoside	-	-	-	+
19	18.5		535	**b** 295.7; 386.8; 516.9; 277.3; 368.8; 219.2	Jaceidin 4'-O-glucuronide	-	-	-	+
20	18.7		471	**b** 163,380,307,205	cumaric acid derivate	+	+	-	-

21	18.9		609	**b** 489, 519, 561, 399, 369, 591	Kaempferol-O-dihexoside	+	+	-	-
22	19		501.7	**b** 193,381,337,178,149	feurilic acid derivate	+	+	-	-
23	19.9		593	**b** 473, 353, 503, 383, 575, 297	Vicinin 2 (isomer)	+	+	-	-
24	20.8		593	**b** 531; 447; 285; 473	Kaempferol-O-(coumaroyl)hexoside	+	-	-	-
25	21		563	**b** 473, 503, 443, 383, 353, 545, 425	Vicinin 1 or 3	+	+	-	-
26	22.2		479	**b** 316.7; 430	myricetin-3-O-glucoside	+	+	-	-
27	22.7		563	**b** 473, 503, 443, 383, 353, 545, 425	Apigenin C-hexoside C-pentoside	+	+	-	-
28	23.2		609	**b** 447, 564, 301	Rutin	+	+	-	-
29	23.6		463	**b** 301	Quercetin-3-O-glucoside	+	-	-	-
30	23.8		753.8	**b** 546.8; 529; 367; 385; 325; 265	1,2-Di-O sinapoylgentiobiose	-	-	-	+
31	24.2		723	**b** 517, 499, 547, 367, 319, 295, 205	2-Feruloyl-1-sinapoylgentiobiose	+	+	-	+
32	24.6		493	**b** 331	6-Methoxyquercetin-7-O-glucoside	+	-	-	-
33	25.2		447	**b** 301	Quercetin rhamnoside (Quercitrin)	+	+	-	+
34	25.4		623	**b** 315, 357, 300, 271, 243	Isorhamnetin 3-O-glucoside 7-O-rhamnoside	+	+	-	+
35	27		432	**b** 285	Cyanidin-3-O-rhamnoside	+	+	-	+
36	27		461	**b** 315, 357, 285, 271	isorhamnetin 3-o-rhamnoside	+	+	-	+
37	27.5	571.5	569.5	**a** 409,303,553 **b** 463; 474.9; 313.4; 417.4	Quercetin derivate	-	-	-	+
38	28.6	555.6	553.6	**a** 409,519,537 **b** 458.8; 447; 313.4; 407; 301; 285	Quercetin derivate	-	-	-	+
39	28.7		583	**b** 477; 489; 420; 327	Quercetin derivate	-	-	-	+
40	29	423	421.3	**a** 317,329,403 **b** 152.7; 135, 315	Hydroxycinnamic acid derivate	-	-	-	+
41	29.8	539.2	537.2	**a** 393,503,521 **b** 431; 391; 442.8; 285	Cyanidin derivate	-	-	-	+
42	29.8	569	567	**a** 423,533,551,409 **b** 461; 421; 327; 473; 519	isorhamnetin derivate	-	-	-	+
43	30.9	555	553	**a** 409,315,537 **b** 406.7; 447; 301	Quercetin derivate	-	-	-	+
44	31.9	539	537	**a** 393,299,521	Cyanidin derivate	-	-	-	+

				b 390.7; 431; 361; 285					
45	31.9		567	**b** 420.7; 461; 315; 392.2	isorhamnetin derivate	-	-	-	+
46	33.8	661.5	659.5	**a** 515,421,409 **b** 583; 641; 526; 433	Quercetin derivate	-	-	-	+
47	33.9		527.8	**b** 240.7; 258.7; 481	Malvidin 3-O-galactoside	-	-	-	+
48	34.6		643.4	**b** 537; 496; 403; 391	Cyanidin derivate	-	-	-	+
49	34.6		673.2	**b** 567; 526; 433; 578.9; 627; 421	isorhamnetin derivate	-	-	-	+
50	34.6		634	**b** 527; 564.6; 347; 240.7	Malvidin derivate	-	-	-	+
51	34.7	415.4		**a** 119; 295; 277; 133: 247: 159	β-Sitosterol	-	-	+	-
52	36.2		587.5	**b** 417; 431; 517; 186.9;	Citrostadienol glucoside	-	-	+	-
53	37.2		661.7	**b** 319.2	Ampeloptin derivate	-	-	+	-
54	38.3		609.8	**b** 315.1; 293; 275; 246	Myricetin derivate	-	-	+	-
55	38.3		625.7	**b** 313; 297	Myricetin Orhamnosylglucose	-	-	+	-
56	38.3		641.5	**b** 313;297; 623	Myricetin derivate	-	-	+	-
57	39.3	425.6		**a** 365; 281; 309	Dihydroisovaltrate	-	-	+	-
58	42	637.4		**a** 581; 525; 469	Tri-galloyl-hexoside I	-	-	+	-
59	42		681	b 653; 447	Quercetin derivate	-	-	+	-
60	42.1		625	**b** 301 (220; 205: 297)	Quercetin O-di-hexoside	-	-	+	-
61	42.6		577	b 277	Sitostanol glucoside	-	-	+	-
62	43.4		629.5	**b** 303(259;205; 285)	Catechin derivate	-	-	+	-
63	43.4		605.5	**b** 303; 279	(epi)Catechin-ethyl dime	-	-	+	-
64	43.6		581.6	**b** 279	Catechin derivate	-	-	+	-
65	44.1		633.8	**b** 305(261,2; 287.1; 207; 233; 177)	Catechin derivate	-	-	+	-
66	44.5		585.8	**b** 281		-	-	+	-
67	45.6	585.4		**a** 479; 493; 387; 303	tetrahydroxyxanthone-di-O,C-hexoside	-	-	+	-
68	47.1	681.6		**a** 663; 561; 543; 545; 411; 393	Unknown	-	-	+	-
69	47.5	663		**a** 607.4; 551; 495; 439	Unknown	-	-	+	-

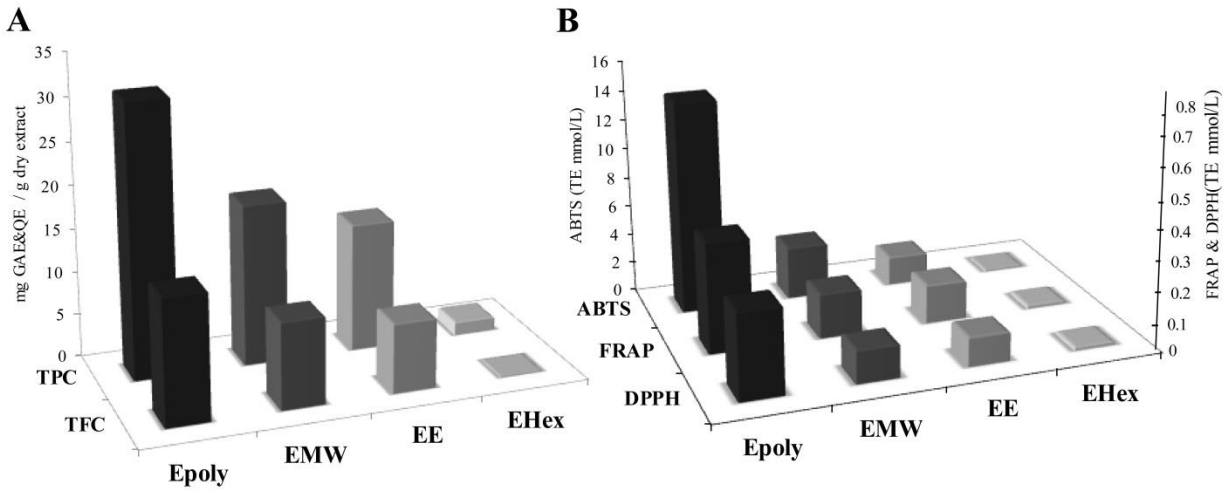
a:MS^2 fragmentation pattern in positive ionization mode; **b**: MS^2fragmentation pattern in negative ionization mode.

Table 2. Total phenolic and flavonoid contents (TPC, TFC) and antioxidant capacity parameters (DPPH, ABTS and FRAP) of *E. foeminea* extracts (n = 3).

	TPC	TFC	DPPH		ABTS		FRAP
	mgGAE/g extract	mgQE/g extract	IC50 mg/mL	TEAC mmol TE	IC50 mg/mL	TEAC mmol TE	TEAC mmol TE
Epoly	**31.5 ± 3.1**	**14.5 ± 0.19**	**0.99 ± 0.059**	**0.286 ± 0.02**	**0.12 ± 0.03**	**14.28 ± 2.7**	**0.37 ± 0.018**
EMW	**18.6 ± 1.2**	**10 ± 0.39**	**3.2 ± 0.069**	**0.11 ± 0.01**	**0.23 ± 0.005**	**3.55 ± 0.09**	**0.15 ± 0.014**
EE	**14.8 ± 1.1**	**8 ± 1**	**4.88 ± 0.11**	**0.097 ± 0.01**	**5.2 ± 0.012**	**1.95 ± 0.07**	**0.13 ± 0.01**
EHex	**1.5 ± 0.5**	**nd**	**>10**	**nd**	**>10**	**nd**	**nd**

TPC: total phenol content; TFC: total flavonoid content; GAE: gallic acid equivalents; QE: quercetin equivalents; IC50: The half maximal inhibitory concentration; TE: Trolox equivalent.

FIGURE LEGENDS



C **Pearson Correlation** (2-tailed)

	TPC	TFC	DPPH	ABTS	FRAP
TPC		*0.99	* 0.97	0.91	* 0.97
TFC	* 0.99		0.93	0.84	0.92
DPPH	* 0.97	0.93		* 0.98	** 0.99
ABTS	0.91	0.84	* 0.98		* 0.98
FRAP	* 0.97	0.92	** 0.99	* 0.98	

Figure 1: Total phenolic and flavonoid contents (TPC, TFC) and radical-scavenging activity of the four *E. foeminea* extracts

(A) Total phenol content (TPC) and Total flavonoid content (TFC) of the four *E. foeminea* extracts were quantified spectrophotometrically and expressed as mg of Gallic acid equivalent per g of dry powder extract (mgGAE/g dry extract), and mg of Quercetin equivalent per g of dry powder extract (mgQE/g dry extract), respectively. **(B)** The antioxidant parameters DDPH, ABTS and FRAP were evaluated by spectrophotemetric assay as described in Materials & Methods Section. Values are reported as Trolox equivalent (mmol TE/L) and IC_{50} . (**C)** Pearson correlation (2-tailed) between TPC, TFC and antioxidant parameters (DPPH, ABTS and FRAP). All values are mean ± S.D from a least three independent experiments. Significant are denoted by symbols: *p≤0.05 and **p≤0.01.

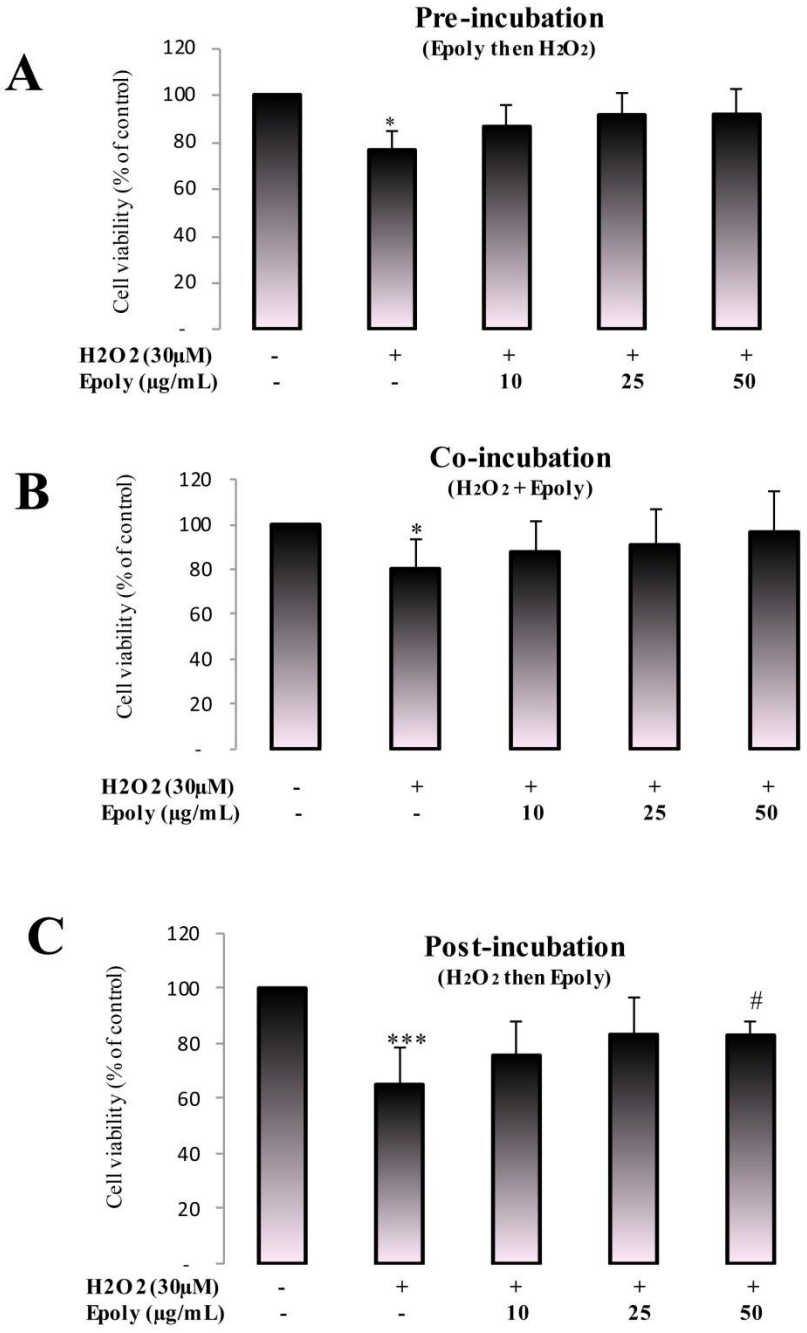


Figure 2: Cytoprotective effect of the *E. foeminea* extracts in H_2O_2-injured HECV cells

Cell viability of HECV cells was assessed by MTT test in the following conditions: **(A)** pre-treatement with Epoly and then exposure to H_2O_2 30 µM for 24h; **(B)** co-treatement with Epoly and H_2O_2 at 30 µM; **(C):** post-treatement with Epoly after incubation with H_2O_2 at 30 µM for 24h. The extracts were used at two concentrations: 25 and 50

µg/mL. Values are mean ± S.D from a least three independent experiments. Significant differences are denoted by symbols: C *vs* H_2O_2 insulted cells ***p≤0.001, *p≤0.05 and H_2O_2 insulted cells *vs* extracts, #p≤0.05.

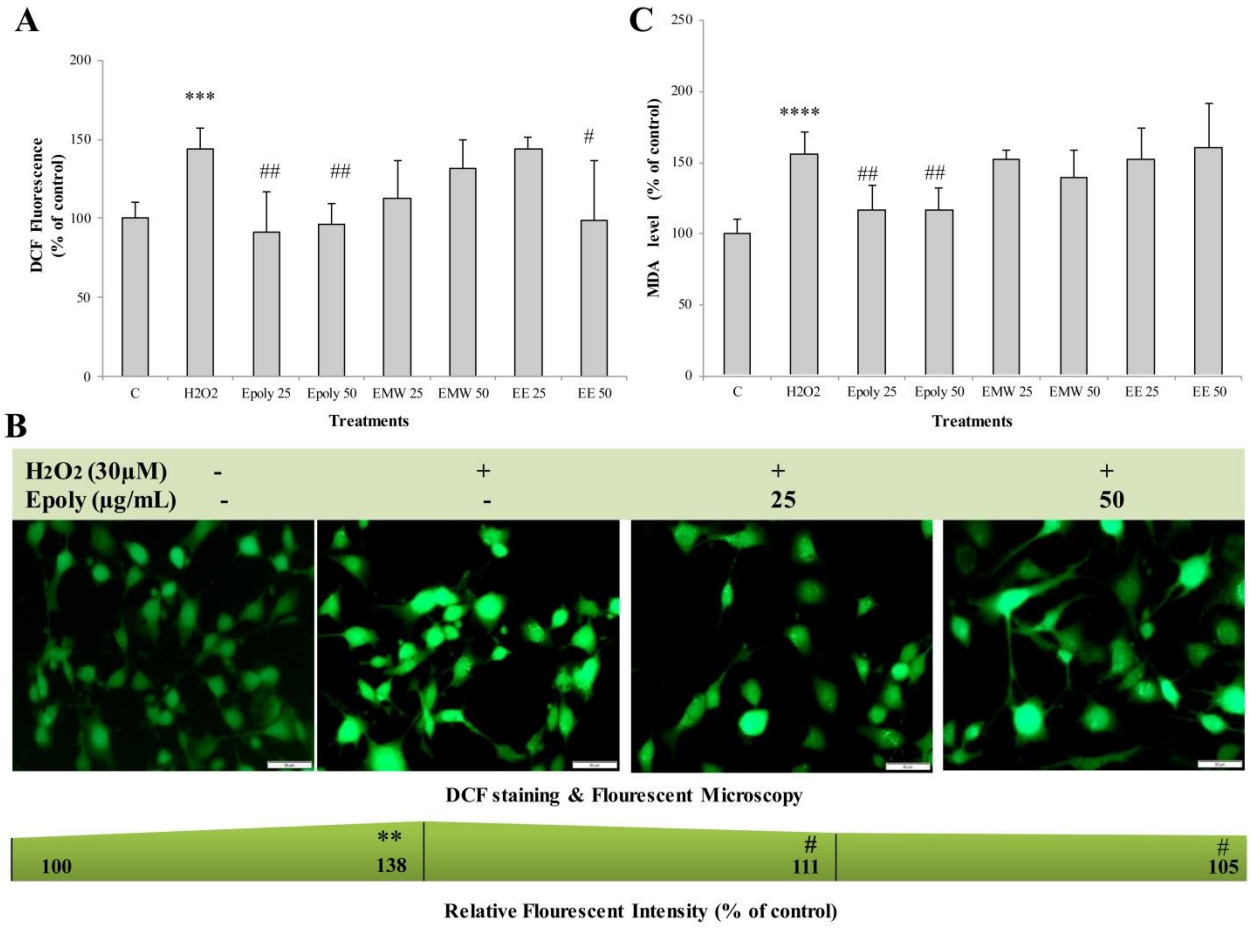


Figure 3: Effects of *E. foeminea* extracts on ROS production and lipid peroxidation in H_2O_2-injured HECV cells

HECV cells were exposed to H_2O_2 (30µM) for 24h, and then treated with extracts Epoly, EMW and EE at two different concentrations (25 and 50 µg/ml) for 24h. Following DCF staining of the cells, the intracellular ROS production was both quantified by spectrofluorimeter assay (**A),** and visualized *in situ* by fluorescence microscopy **(B).** Images were acquired at Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany), magnification 20x (Bar: 20µm). (**C)** Intracellular MDA level was quantified by TBARS assay as pmol MDA/mL x mg of sample protein. Values are reported as % of control and are mean ± S.D from a least three independent experiments. Significant differences are denoted by symbols: C *vs* H_2O_2 insulted cells **p≤0.01, *p≤0.05 and H_2O_2 insulted cells *vs* different concentration of extracts, ### p≤0.001, ##p≤0.01, #p≤0.05.

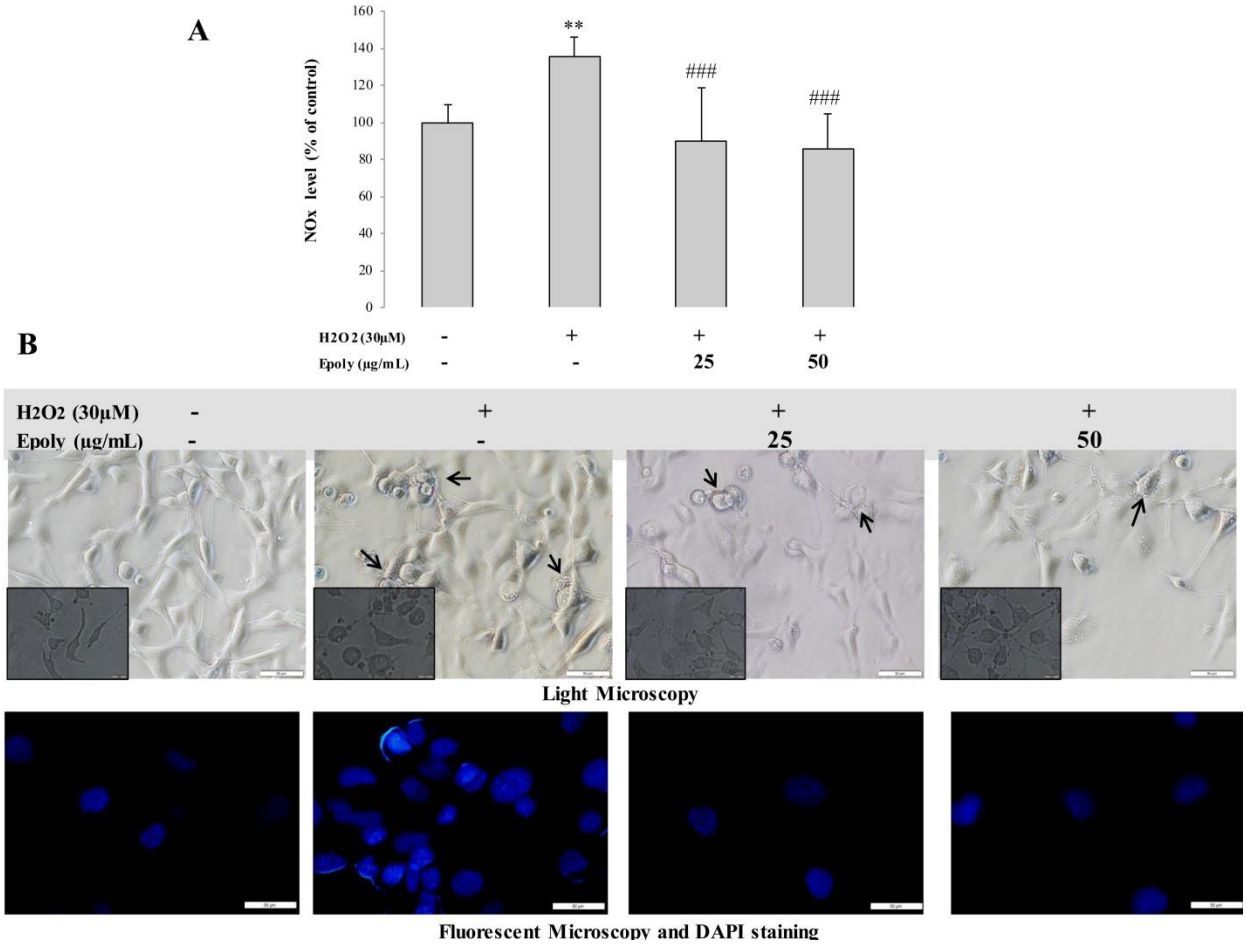


Figure 4: Cytoprotective effect of the *E. foeminea* extract Epoly on cellular and nuclear morphology and NO release H_2O_2 injured HECV cells

HECV cells were treated with H_2O_2 (30µM) for 24h, and then were treated with E poly at 25 and 50 µg/ml concentrations for 24h. The following parameters were evaluated: (**A)** Nitric oxide production quantified in the medium of HECV cells as µmol $NaNO_2$/mg sample protein by Griess reaction. Values are expressed as % of control. Values are mean ± S.D from a least three independent experiments. Significant differences are denoted by symbols: C *vs* H_2O_2 insulted cells **p≤0.01, and H_2O_2 insulted cells *vs* different concentration of E poly extract, ### p≤0.001; (**B)** Representative images of HECV cells observed under a Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Magnification 20x; Bar:50µm; 40x; Bar:20µm). The presence of micronuclei was assessed by Fluorescence Microscopy of DAPI-stained cells under an inverted Olympus IX53 microscope equipped with a CCD UC30 camera and (Magnification 40x; Bar: 20µm).

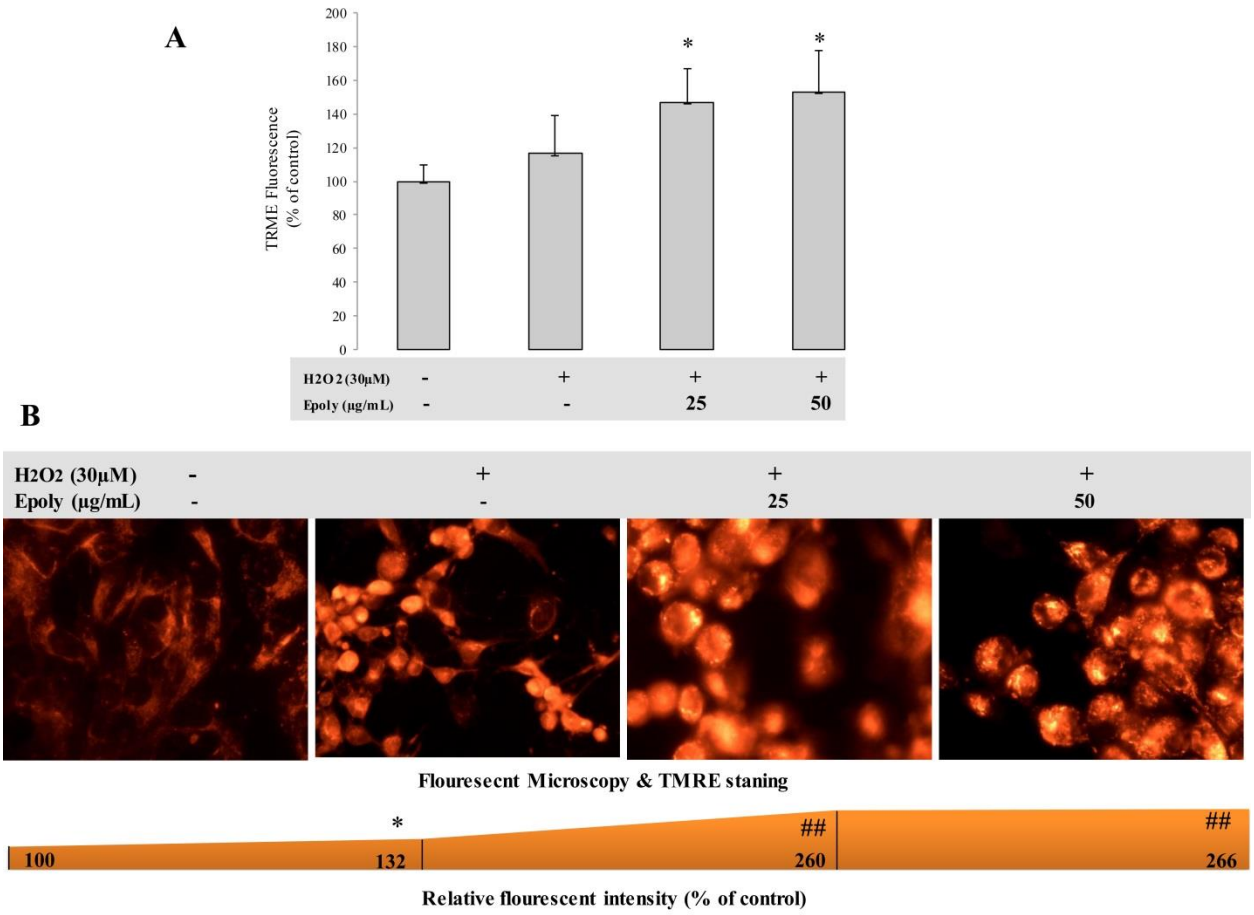


Figure 5: Effect of the *E. foeminea* extract Epoly on mitochondrial membrane potential in H_2O_2-injuredd HECV cells

HECV cells treated with H_2O_2 for 24,h and then with E poly (25 and 50 µg/ml) for 24h, were assessed for the mitochondrial membrane potential through TMRE-staining of living cells. **(A)** TMRE fluorescence was quantified by spectrofluorimeter assay. Values are expressed as % of control. Values are mean ± S.D from a least three independent experiments. Significant differences are denoted by symbols: H_2O_2 insulted cells *vs* different concentration of E poly extract, * $p \leq 0.05$. (**B)** Intracellular TMRE fluorescence was visualized *in situ* by fluorescence microscopy and image analysis using the free software ImageJ. Images were acquired at Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany), magnification 20x (Bar: 20µm).

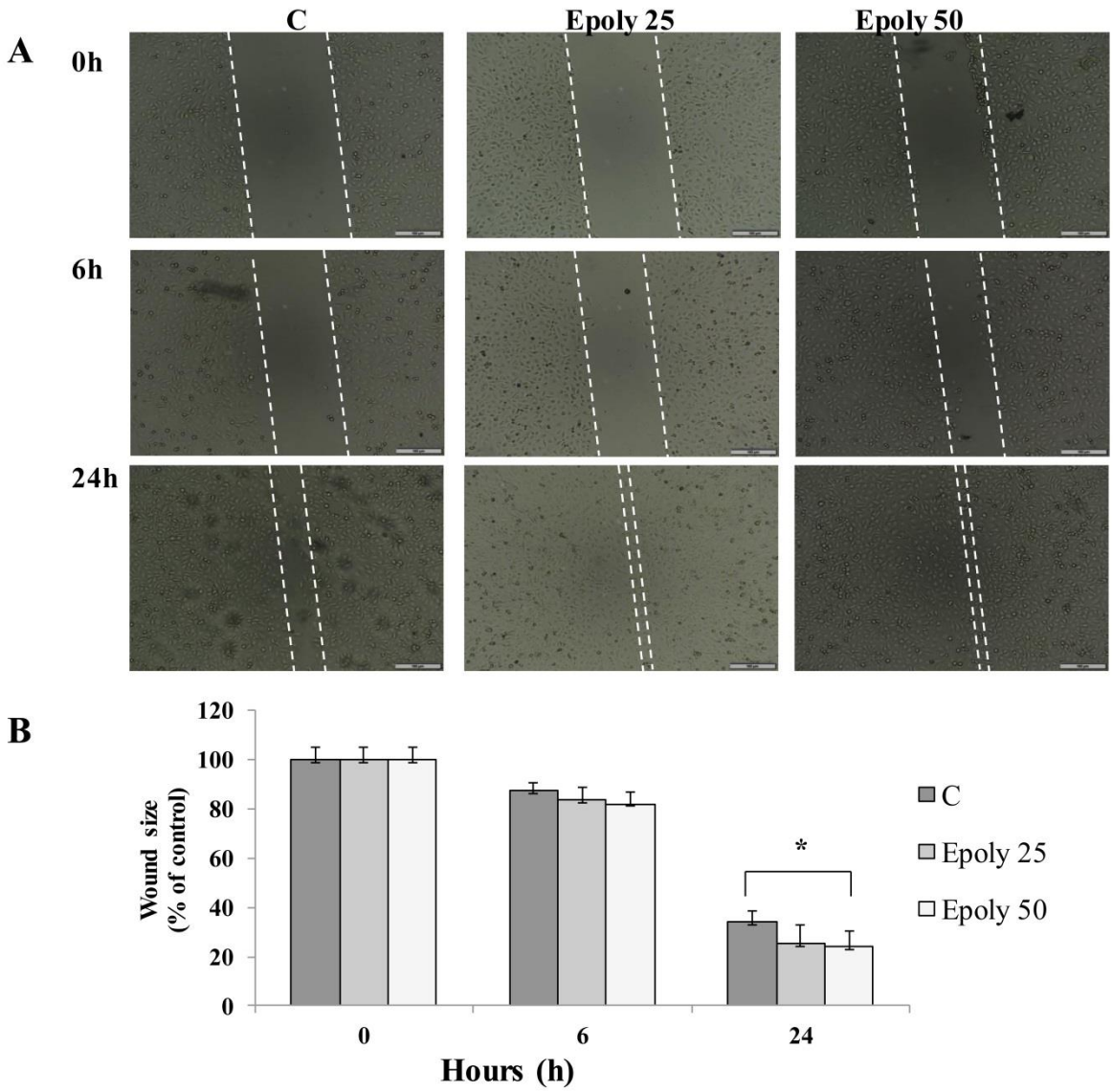


Figure 6: Effect of *E. foeminea* extract Epoly on endothelial cell migration and wound repair

HECV cells treated with H_2O_2 for 24 h, and then with E poly (25 and 50 µg/ml) for 24h were subjected to wound healing assay as described in Materials and Methods. Images were acquired at 0, 6 and 24 h from the beginning of the assay and the wound area was calculated by ImageJ software. Images were acquired at Leica DMRB light microscope equipped with a Leica CCD camera DFC420C (Leica, Wetzlar, Germany), magnification 20x (Bar: 20µm). (**A)** Representative images are shown from three independent experiments and the dotted lines define the areas lacking cells (wound area). (**B)** Graphs representing the changes (percentage) of the wound area during the time. The wound area was expressed as % of control at time=0. Values are mean ± S.D from at least three independent experiments. Significant differences are denoted by symbols C *vs* Epoly treatment *p≤0.05.

➢ DISCUSSION

Use of pharmaceutical drugs, often derived from medicinal plants, has largely replaced herbal treatments in modern health care. However, many people continue to employ various herbs in traditional or alternative medicine. Flora of the Mediterranean area includes many edible species that have been used in traditional medicine and have always represented an important source of bioactive compounds. In Lebanon, folk medicine is widely diffuse and the region is rich in medicinal plants.

Since oxidative stress plays a crucial role in many chronic diseases such as Cancer, Liver and neurodegenerative pathologies, considerable effort has been made to find natural and non-toxic antioxidant compounds that prevent/treat these diseases and counteract their progression. In fact, numerous plant-derived secondary metabolite especially polyphenols (PC) from medicinal plants possess radical scavenging/antioxidant activity, especially when studied in cell-free systems as well as in cells *in vitro*, or even *in vivo*. These polyphenols contribute to the beneficial effects of medicinal plants; however, the exact molecular target for many PC is still unclear. (Willcox et al., 2004; Chen et al., 2016)

In this context, my work during the PhD course aimed to investigate some common medicinal plants typically employed in Lebanon as food or in folk medicine in terms of bioactive compounds and possible beneficial effects.

My study focused on three plants typical of the East Mediterranean area: *Thymbra spicata,* an aromatic plant of the *Lamiaceae* family (called "*Za'atar*" in Lebanon); *Ephedra foeminea* a non-flowering shrub of the *Ephedraceae* family (called "*Alanda*" in Arab); *Rhus coriaera* an herbal shrub of the *Anacardiaceae* family (called "*Sumac*" in Lebanon). These plants are wildly growing in Lebanon, and for my study these plants have been collected from "*Maarake*" in South Lebanon. From each plant we prepared and tested different extracts using different solvents in order to recover specific classes of compounds.

The leaves of *T. spicata* are rich in phenolic compounds which received growing attention among nutritionists as possible prevention for many metabolic disorders, especially those where oxidative stress is involved. From the aerial parts of *T. spicata* we prepared two extracts using ethanol and water as solvent. The ethanolic extract was richer in volatile compounds and had

higher phenol/flavonoid content than the aqueous extract. Carvacrol and rosmarinic acid were the most abundant compounds in the ethanolic and the aqueous extract, respectively.

- In the first study, we tested the possible protective effect of *T. Spicata* extracts at low doses (from 0.15 to 15 µg/mL) on an *in vitro* model of hepatic steatosis widely employed in previous studies of our group; it consists of rat hepatoma FaO cells exposed to a mixture of oleate/palmitate. Both extracts showed a good lipid lowering activity. However, the aqueous extract was more effective to counteract the hepatic steatosis *in vitro* than the ethanolic extract. The lipid-lowering activity of the extracts was depending on their action on lipid droplet whose number and size were reduced. Excess fat accumulation in hepatic cells is typically accompanied by increased oxidative stress. As markers of oxidative stress we assessed both the intracellular ROS production, and the level of lipid peroxidation. Both these indices were significantly increased in steatotic cells compared to control hepatocytes, and reduced by both the extracts. However, the ethanolic extract was most effective as anti-oxidant than the aqueous extract. NAFLD and obesity are often associated to endothelium dysfunction. Therefore both *T. spicata* extracts were also tested on HECV human endothelial cells after exposure to an oxidant insult (H_2O_2). The condition of oxidative stress was partially counteracted by both extracts, but the ethaiolic extract was more effective to protect against oxidative stress than the aqueous one. Both the extracts were also evaluated for their effects on endothelial cell migration. We observed that both extracts were able to accelerate the wound repair, but also in this case TE was more effective than TW. In conclusion, our results on *T. spicata* extracts show that at low dose (1.5 µg/mL) they play beneficial effects as they are efficacious as lipid-lowering and antioxidant agents for steatotic hepatocytes and dysfunctional endotheliocytes, two typical alterations occurring during NAFLD.

  In a second study, we tested if the *T. spicata* extracts at higher doses (50 and 100 µg/mL) might have antitumor potential. We demonstrated that ethanolic extract from *T. spicata* plays significant *in vitro* antitumor activity on different cancer cell lines, likely depending on the pool of its phenolic components. Our findings showed that aqueous extract did not play cytotoxic activity on nabny cancer cell line and that ethanolic extract was more cytotoxic than pure carvacrol, its major component, likely depending on the synergism

with some other phenolic compounds being present in the extract. To clarify the mechanisms of TE activity we focused on MCF-7 (a breast cancer cell line). We found that TE exerted long term antitumor effects on MCF-7 cells through inhibition of cell migration, colony formation, and of cell proliferation recovery after treatment removal. Both TE and carvacrol exerted effects on ROS generation and NO release, this effects were significantly more efficient for TE than carvacrol these combined actions (generation of ROS/RNS) led to a stronger pro-apoptotic activity of TE compared to carvacrol resulting in the appearance of a late-apoptotic phenotype. NFκB and STAT3 are inflammation-linked transcription factors which modulate the ability of malignant cells to elude tumor-surveillance, and promote tumor angiogenesi. Our findings show that TE inhibited the NFκB signaling in MCF-7 cells through downregulation of phospho-p65, and this action may be a mechanism for suppressing metastasis of breast cancer cells. Moreover, we found that TE inhibited STAT3 phosphorylation, and this effect might be related to the pro-apoptotic effect of TE.

In conclusion, *T. spicata* is rich of poly(phenols) such as carvacrol, Rosmarinic acid and flavonoids, low dose of these polyphenols, exert a high radical scavenging activity and play a potential antioxidant and antisteatoitc activites in an *in vitro* cellular model, these polyphenols defined as exogenous reducing compounds, may act interactively (e.g., synergistically) with endogenous antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH) to maintain or re-establish redox homeostasis. In other hand, high doses of these compounds resulted cytotoxic in cancer cells model, which can promote cell death by apoptosis and inhibit cell proliferation and migration in Breast cancer cell line, this effect may due to the prooxidative effects at high concentrations of these polyphenols which led to cell death. This double-edged effect of polyphenols (anti and pro oxidant) depends of many factors especially concentration. In fact, in line with our findings, many studies employing cell models have highlighted the cytoprotective activity of plant food constituents such as polyphenols and mixtures (already known as antioxidants such as quercetin, catechins including epicatechin and epigallocatechin-3-gallate (EGCG) and gallic acid) and their preventive effects against oxidative stress-induced cell death and dysfunction, and also they can display prooxidant activities under certain conditions, such as at high doses or in

the presence of metal ions (Sergediene et al., 1999; Galati and O'Brien, 2004; Watjen et al., 2005; Robaszkiewicz et al., 2007; De Marchi et al., 2009).

- *Ephedra* genus is one of the most ancient medicinal plants, *E. foeminea*, a non-flowering shrub of this family, finds some application in traditional medicine in Lebanon. Despite the popularity of the traditional use, there is little information about its bioactivity and phytochemicals constituents. In our study, four extracts were prepared form fruits of *E. foeminea*, using different extraction solvents to obtain a large profile of their chemical compositions, using solvent with different polarity such as ethanol, Methanol/Water and hexane, in addition, another optimized methods for water soluble polyphenols extraction was also performed to cross the water solubility obstacle in pharmacological researches. A total of 69 phenolic compounds were characterized in all extracts by HPLC/MS analysis. Most of them were reported for the first time in *E. foeminea* and some were reported for first time in *Ephedra* genera. In addition, the polyphenolic extract presented the higher total phenols/flavonoids content in comparison to other extracts (which reflect the efficiency of polyphenolic extraction method). Concurrently, the polyphenolic extract exhibited stronger antioxidant capacity and higher radical scavenging activity. Quercetin rhamonside was the major compound found in the extracts by HPLC/MS analysis, the flavonol Quercetin and his conjugate and derivate had a wide range of applications in pharmacological processes and they are well known in their vast biological activity.

  Therefore, our results are of interest to further studies on the phytochemical composition of *E. foeminea* and the *Ephedra* family. Additionally, we explored also the biological activates in an *in vitro* cellular model of oxidative stress and atherosclerosis consisting on human endothelial cells HECV injured with free radicals, we studied the potential antioxidant effect of *E. foeminea* extracts. Exposure HECV cells to hydrogen peroxide H_2O_2 promotes oxidative stress and cell death (mainly by apoptosis). Besides, the water-soluble polyphenolic extract Epoly, showed a significant antioxidant activity against H_2O_2-induced oxidative stress and protected HECV cells from apoptosis and cell death. In details, the oxidative stress expressed by ROS and lipid peroxidation induced in HECV

cells by exposure to H_2O_2 was counteracted by Epoly treatment at 25 and 50 µg/mL, as well as Nitric oxide NO release was reduced after treatment with Epoly.

In addition we observed a significant increase of in mitochondrial membrane potential after treatment of Epoly with respect to H_2O_2-insulted cells. The extracts were also assessed for their effects on endothelial cell migration and wound healing capability. We observed that Epoly at 25 and 50 µg/mL was able to accelerate the wound repair in HECV cells.

Taken together, our results show the potential of polyphenols from *E. foeminea* fruits as sources of natural antioxidants exerting protective effects on HECV cells against free radical-induced damage. Such effect is may related to a direct scavenging activity of polyphenols towards free radicals and also to the modulation of pivotal intracellular mechanisms such as mitochondrial antioxidant defense system. However, our results clearly demonstrate that the intracellular antioxidant response activation is the main mechanism accounting the protective effect of Epoly by the reduction of intercellular ROS level, lipid peroxidation and NO release as well as the inhibition of H_2O_2-induced cell death and apoptosis. To the best of our knowledge, this work is the first attempt for investigating the detailed chemical compositions of *E. foeminea* fruits and the cytoprotective mechanisms mediated by polyphenols against the damage induced by free radicals in cell cultures. Establishing the differences in the prevalence of protective mechanisms induced by polyphenols may be useful in the design of novel strategies to maximize the efficiency of healthy effects mediated by dietary polyphenols.

- *Rhus Coriaria* is a wild edible plant growing in the Mediterranean region. The dried fruits are the most commonly consumed part of this plant which is typically used as a condiment, spice, sauce, and appetizer. In folk medicine this plant has been used in the treatment of many disorders. Furthermore, many *in vitro* and *in vivo* studies suggest that *R. coriaria* might possess many pharmacological activities. The effect of *R. coriaria* on microglial inflammation has not been reported before. In the present study, we studied the possible anti-oxidant and anti-inflammatory capacity of *Rhus Coriaria* fruits on microglial cells (BV-2) exposed to H_2O_2 and LPS as *in vitro* cellular model for oxidative stress and inflammation. Two extracts were prepared by solvent extraction using ethanol

and water to obtain the ethanolic extract RE and aqueous extract RW. The phytochemical screening revealed a high variety of secondary metabolites especially polyphenols and flavonoids which they present in high amount. Due to the presence of high content in phenolic compounds in both extracts, consequently, they showed a high radical scavenging activity assessed by DPPH assay (in comparison of ascorbic acid used as positive control). The *in vitro* evaluation of their biological activity was assessed by using Microglial BV-2 cell lines as cellular model; these cells were exposed to H_2O_2 and LPS in order to induce oxidative stress and inflammation, respectively. We found that both extracts decreased the expression of TNFα (by RT-PCR mRNA quantification) and NFκβ (by western Blot protein quantification), as major pro-inflammatory marker. Besides, both extracts increased the expression of IL-10 (by RT-PCR mRNA quantification), an anti-inflammatory cytokine. In addition, both extracts exert an anti-oxidant property against H_2O_2-induced oxidative stress in BV-2 cells by the reduction of ROS level and the recovery of cell viability after H_2O_2 exposure, which indicate the cytoprotective effects of extracts against H_2O_2-induced cytotoxicity in BV-2 cells.

Collectively, present study investigated the antioxidant and antinflammatory capacity of extracts of *Rhus Coriaria* fruits and its protective effect against H2O2-induced oxidative damage in BV-2 cells. Likely depending of high amount of polyphenols found in both extracts, and due to their capability do scavenge free radicals, these extracts had a potential anti-oxidant and anti-inflammation activities in Microglial cells. It could conclude that cytoprotective effect of *Rhus Coriaria* depends on scavenging ROS and modulation of endogenous antioxidant systems Overall, these results contribute to explaining the past and current usage of *Rhus Coriaria* in folk medicine, as fruits extracts can be regarded as a promising source of antioxidant phenolic compounds for further uses in pharmacology. Inflammation and oxidative stress processes in Microglial cells are strictly related with several neurodegenerative diseases such as Alzheimer, and natural compounds, present in this medicinal plants, could be a valuable tools for new therapy/co-therapy for these diseases.

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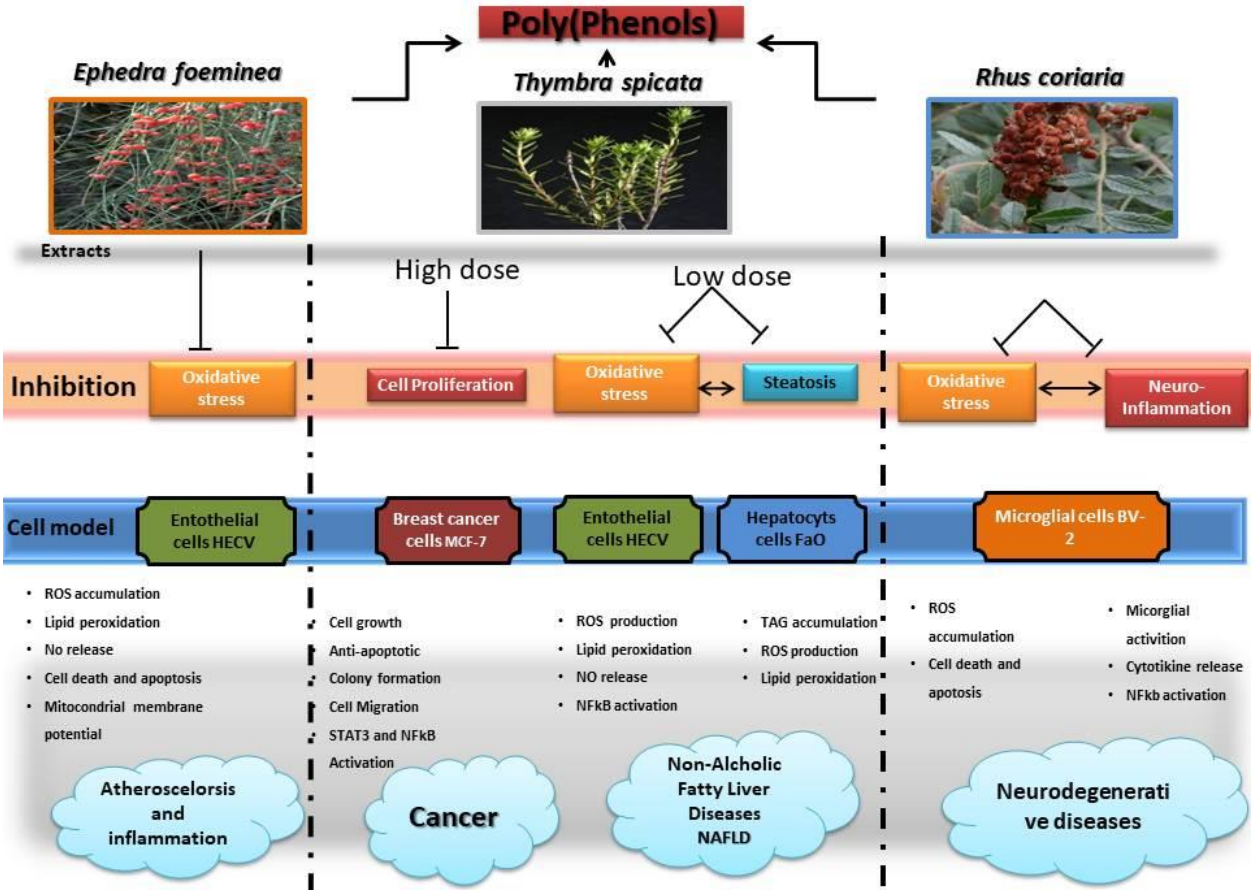
➢ Conclusions

Many reports suggest that having chronic diseases is associated with herbal medicine use. The use of high valuable medicinal plants in Lebanon is well documented, it is considered as home remedies and first choice of safer and effective treatment of many illnesses. Sinice that there are no enough scientific reports and information about the chemical compostion and bioactivity of some plants, and in term of supporting the tradional use by scientific evaluation, the present work provides valuable information on the bioactivities of different plant species from the Lebanese flora, which are widely used for medicinal and culinary purposes, in relation to aspects of antioxidant, anti-inflammatory, anti-steatotic and antitumor activities. Summarizing all data here obtained, it can be concluded that *Thymbra spicata, Epherdra foeminea* and *Rhus coriaria* examined in our study contain valuable biologically active components, such as Polyphenols which may responsible of their biological effects with considerable antioxidant abilities of extracts of these plants have been showed; this is probably due the high content of phenolic compounds determined in these samples. These polyphenols showed beneficial effects in *in vitro* cellular model of different diseases such as NAFLD, inflammation and atherosclerosis. In addition, *T. spicata* ethanolic extract which resulted in high content in phenolic compounds especially Carvacrol (CVL) showed a good antitumor activity in Breast cancer cells, the high dose of this extract acts as prooxidant and resulted to promote apoptosis and cell death and interestingly with minor cytotoxicity in non-tumor breast cells. Our data suggest that these species may have a great relevance as healthy supplements in the prevention and therapies of diseases in which free radicals and oxidants are implicated.

The interest of research in medicinal plants filed is growing. Traditional medicine based on medicinal plants use, represents an important strategy for the health systems worldwide to treat and prevent many diseases. Despite the extensive experiences in use of medicinal plants in traditional medicine, scientific study and identification of bioactive plant compounds and their activity on biological systems can promote the discovery of new therapeutic plant-based agents in the future. In this context, extensive research is fundamentally needed to isolate and characterize new bioactive natural compounds and use appropriate biological systems to evaluate the bioactivity, target and possible toxicity, and then, animal studies and clinical trials are required to explore the physiological effect of these plants.


Poly(Phenols)
Ephedra foeminea
Thymbra spicata
Rhus coriaria
Extracts
High dose
Low dose
Inhibition
Oxidative stress
Cell Proliferation
Oxidative stress
Steatosis
Oxidative stress
Neuro-Inflammation
Cell model
Entothelial cells HECV
Breast cancer cells MCF-7
Entothelial cells HECV
Hepatocyts cells FaO
Microglial cells BV-2
ROS accumulation
Lipid peroxidation
No release
Cell death and apoptosis
Mitocondrial membrane potential
Cell growth
Anti-apoptotic
Colony formation
Cell Migration
STAT3 and NFkB Activation
ROS production
Lipid peroxidation
NO release
NFkB activation
TAG accumulation
ROS production
Lipid peroxidation
ROS accumulation
Cell death and apotosis
Micorglial activition
Cytotikine release
NFkb activation
Atheroscelorsis and inflammation
Cancer
Non-Alcholic Fatty Liver Diseases NAFLD
Neurodegenerative diseases